STRUCTURAL AND FUNCTIONAL STUDIES OF REOVIRUS RNA-DEPENDENT RNA POLYMERASE COMPLEX

By

Peng Yin

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

Doctor of Philosophy

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DEDICATION

I would like to dedicate this thesis to my wife, Juanjuan Yang Yin and my daughter, Jessica Hua Yin for their loves and supports which have provided me the strength and courage during this study

This is for you

Acknowledgments

I would like to thank a number of people who helped me and made this work possible.

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Finally, but most importantly, I would like to thank my wife, my daughter and my parents, my sisters in China who always believe in me, support me for all these years.

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ABBREVIATIONS

CPE: cytopathic effect;

DAB: 3.3'-diaminobenzidine;

DEPC dH_2O: diethylpyrocarbonate-treated dH_2O;

DDT: dithiothreitol;

DMSO: dimethyl sulfoxide;

DOC: deoxycholate

Freon: trichlorotrifluoroethane;

[^{3}H]SAM: 8-azido-S-adenosy-[^{3}H]methionine

Hepes: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid];

HRP: Horse Radish Peroxidase;

ISVP: Intermediate Subviral Particle;

MEM: modified minimal essential medium;

MOI: multiplicity of infection;

PBS: phosphate-buffered saline;

PFU: plaque forming unit;

PMSF: p-phenylmethanesulfonyl fluoride

Ponceau S: 3-hydroxy-4-[2-sulfo-4-(4-sulfophenylazo) -phenylazo]-2,7-naphthalenedisulfonic acid;

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel;

T1L: reovirus serotype 1 Lang;

T2J: reovirus serotype 2 John;

T3D: reovirus serotype 3 Dearing;

TCA: trichloroacetic acid;

ABSTRACT

The reovirus core, formed by five different structural proteins and 10 segments of dsRNA, is a multienzyme complex. It contains all necessary components for transcription, methylation, and capping of progeny mRNA. The reovirus transcriptase has an unusual temperature profile, with optimum transcription occurring at approximately 50°C, and little activity occurring below 30°C or above 60°C. Purified type 1 Lang cores transcribed most efficiently at 48°C. The transcriptase temperature optimum of purified type 3 Dearing cores was 52°C. In addition, T1L cores produced more mRNA per particle than did T3D cores at their respective temperature optima. Core particles were purified from inter-typic reassortants and used to map these differences. The M1 gene, which encodes minor core protein $\mu 2$, was uniquely associated with the difference in temperature optimum of transcription ($P = .0003$). The L1 gene, which encodes minor core protein $\lambda 3$, previously implicated as the RNA polymerase, and the M1 gene were associated with the difference in absolute amount of transcript produced ($P \leq 0.01$ and $P \leq 0.0002$ respectively). These data suggest that minor core protein $\mu 2$ also plays a role in reovirus transcription.

I also found that reovirus transcriptase within core has little activity occurring above 60^0C. However, cores preheated at 65^0C can still transcribe after return to 48^0C,

suggesting a reversible alteration in the transcriptase complex, while the enzymatic activities of cores heated at 70°C are irreversibly lost. Morphologically, cores heated at 65°C or 70°C have significantly fewer, or no, λ2 spike structures. Approximately 50% of the spike-defective cores treated at 65°C have genomes, but none of the cores heated at 70°C have genomes. The transcripts synthesized by cores preheated at temperature up to 65°C, then re-incubated at 37 or 48°C for 1 hour, are viral ssRNA molecules as shown by hybridization assays. Protein analyses and protease sensitivity assays of heated cores indicated that the λ2 spikes have undergone dramatic conformational alterations; the spike structures and λ2 proteins were completely removed by the heat and protease digestion treatments. *In vitro* translation assays demonstrated that viral proteins can be translated from the ssRNAs produced by 65°C heated cores, as well as by the cores treated with heat (65°C) and chymotrypsin treatments. These observations suggest that the spike-deficient cores still possess transcriptase activity. Since previous attempts to partially disassemble the core structure completely destroyed its enzymatic activities, these data represent the first system capable of partially dissociating reovirus cores without destroying their transcriptase activities and provide a novel system for better understanding of an important enzymatic complex.

1. INTRODUCTION

The name, reovirus (respiratory, enteric, orphan viruses) was first introduced in 1959 (Sabin, 1959), since it was originally isolated from human respiratory and gastrointestinal tracts and not associated with any known diseases. Reovirus is the prototype of the family *Reoviridae* which is currently divided into nine genera. Four of these genera: Reoviruses, Orbiviruses, Rotaviruses and Coltivirus infect animals, including humans. The other genera, such as Cypovirus, Phytoreovirus and Fijivirus are classified together, because of their similar structures, genomes, replicative strategies and host ranges, infecting only plants and insects (Mattews, 1979; Joklik, 1983).

1.1. Reovirus structure

The mammalian reovirus is a non-enveloped, spherical viral particle 85nm in diameter (Fig. 1). It consists of 10 double-stranded RNA segments, enclosed in a double protein shell(for reviews see references (Nibert *et al.*, 1991, 1996). The outer shell is formed by two major proteins, μ1 (encoded by the M2 gene) and σ3, (encoded by the S4 gene) and a minor protein σ1, cell attachment protein (encoded by the S1 gene) (Smith *et al.*, 1969; Bassel-Duby *et al.*, 1985, 1986). The inner capsid (core) is composed of two major proteins, λ1

(encoded by the L3 gene) and σ2, (encoded by the S2 gene) and two minor proteins, λ3 (encoded by the L1 gene) and μ2 (encoded by the M1 gene) (Matsuhisa and Joklik, 1974; White and Zweerink, 1976; Xu *et al.*, 1993). Ultrastructural studies suggested the twelve spike structures are extended from inner core to surface of outer shell on twelve 5-folded vertices of reovirus (Luftig *et al.*, 1972). These findings were later confirmed by immunological studies in which antibodies specifically against λ2 could react to virions (Hayes *et al.*, 1981; Lee *et al.*, 1981; Virgin *et al.*, 1991). The λ2 proteins (encoded by the L2 gene) are assigned to be the components for these spikes, because spikes could be selectively removed from core structure by high pH treatment and protein λ2 is absent from remaining core structure (White and Zweerink, 1976). In addition, cross-linking studies of removed λ2 proteins by high pH treatment estimated that each spike is composed of five λ2 proteins (pentamers) (Ralph *et al.*, 1980).

There are three distinct types of reovirus particles, virion, Intermediate Subviral particle (ISVP) and core particle which exist both *in vivo* and *in vitro* (Fig. 1, Table 1). The ISVP and core particles can be generated *in vitro* by digesting virions with proteases (chymotrypsin or trypsin). When virions are converted to ISVPs, outer capsid protein σ3 is removed from virion and μ1/μ1C are cleaved to fragments of δ and φ. In addition, cell attachment protein σ1 undergoes comformational change from folded form to extended form. For

conversion to cores, further cleavages and removal of outer capsid proteins are required (σ1, δ and ϕ). The virions and ISVPs are infectious but not transcriptionally active. By contrast, core particles are non-infectious, but transcriptionally active. The core is a multiple enzyme complex that contains all necessary components for transcription, methylation, and capping of progeny mRNA (Chang and Zweerink, 1971; Shatkin, 1974; Furuichi *et al.*, 1975). Previous studies indicated that antibody directed against protein λ2 could react with virions, ISVPs and cores (Hayes *et al.*, 1981; Lee *et al.*, 1981; Virgin *et al.*, 1991). In addition, when reovirus virions or core particles were treated with ^{125}I (iodine-125), protein λ2 was the only protein labelled on both types of the particles (White and Zweerink, 1976). This evidence suggests the pentameric spikes are exposed on surface of virions, ISVPs and cores. However, cryoelectron microscopy and computer reconstruction demonstrated these pentameric spike structures in the cores are significantly different from those in virions and ISVPs. A channel 8nm in diameter in each pentameric spike can be observed in cores, but not in virions and ISVPs (Dryden *et al.*, 1993). Previous studies indicated that reovirus ssRNAs are synthesized within core particles (Chang and Zweerink, 1971; Silverstein *et al.*, 1972). Therefore, these channels only seen in cores may serve as a pathway for exit of viral mRNAs from inside the cores to the cytoplasm. Comparisons of

virion, ISVP and core properties are summarized in Fig. 1 and Table 1.

1.2. Reovirus dsRNA genome

Both cryoelectron microscopy and low-angle x-ray diffraction showed that the reovirus genome is tightly located in the centre of the virion in a well ordered fashion (Harvey *et al.*, 1981; Dryden *et al.*, 1993). Physiochemical properties of the reovirus genome such as high and sharp melting temperature, lower buoyant density in Cs_2SO_4 than ssRNA, acridine orange stained reovirus-infected cells, relatively resistant to RNase I and the same purine:pyrimidine composition all suggested that reovirus contains a dsRNA genome (Gomatos *et al.*, 1962; Gomatos and Tamm, 1963; Bellamy *et al.*, 1967). In addition, electron microscopy, sedimentation and chromatography indicated that the reovirus genome is segmented and can be classified into three sizes (Gomatos and Stoeckenius, 1964; Bellamy and Joklik, 1967; Dunnebacke and Kleinschmidt, 1967; Watanabe and Graham, 1967; Vasquez and Kleinschmidt, 1968; Lyubchenko *et al.*, 1992). Moreover, polyacrylamide gel electrophoresis clearly separated and demonstrated these 10 gene segments: three large (L1, L2, L3), three medium (M1, M2, M3) and four small (S1, S2, S3, S4) gene segments (Shatkin *et al.*, 1968; Watanabe *et al.*, 1968). The eleven correlated viral proteins, including eight

Figure 1

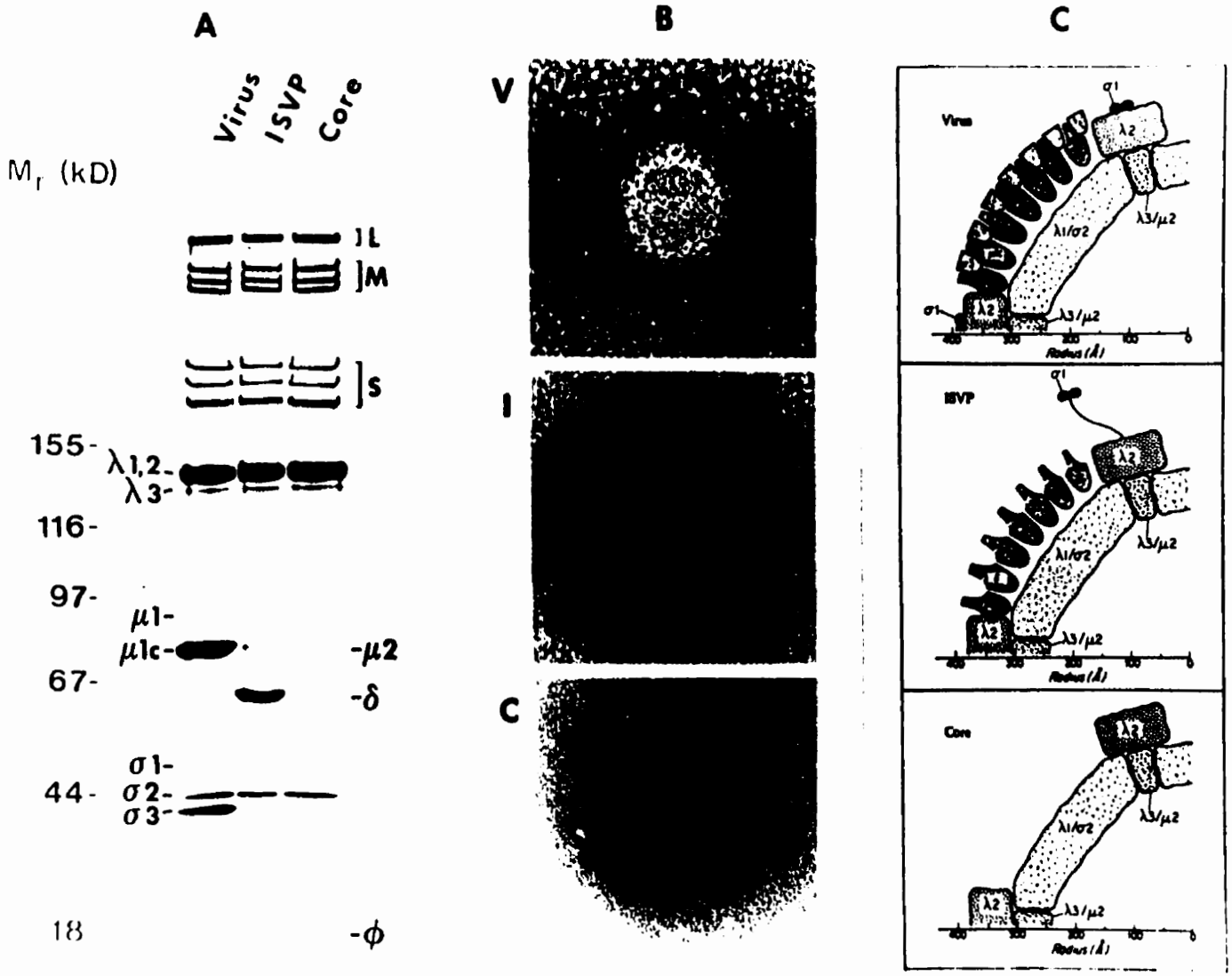

A
B
C
Virus
ISVP
Core
M_r (kD)
]L
]M
]S
155-
λ1,2-
λ3-
116-
97-
μ1-
μ1c-
-μ2
67-
-δ
σ1-
44-
σ2-
σ3-
18
-φ
V
I
C
Virus
ISVP
Core
σ1
λ2
λ1/σ2
λ3/μ2
Radius (Å)

Fig. 1. The reovirus virion, ISVP and core resolved in SDS-PAGE (A) or observed by negative stain transmission electron microscopy (B), and diagrams of virion, ISVP and core based upon known information (C). In (A), three sizes of 10 dsRNA gene segments labelled as large (L), medium (M) and small (S) are in upper part of the gel. The viral proteins are in lower part of the gel as labelled. In (B), the electron microscopy pictures of virion (V), ISVP (I) and core (C) from top to bottom. Notice the slight difference in sizes of the three particles. The fibers only seen in ISVP are believed to be fibrous forms of the cell attachment protein σ1. The diagrams in (C) show the different sizes of three particles, different compositions of viral proteins for virion, ISVP and core and possible positions and relations of each structural proteins in virion, ISVP and core. Proteins μ1 and σ3 may be associated and compose the outer shell, along with cell attachment protein σ1. The inner capsid (core) is composed of two major proteins (λ1 and σ2) and two minor proteins (λ3 and μ2). The spike structures formed by λ2 proteins (White and Zweerink, 1976) are exposed on surfaces of virion, ISVP and core (White and Zweerink, 1976; Hayes *et al.*, 1981; Lee *et al.*, 1981; Virgin *et al.*, 1991).

Table 1. Characteristics of virions, ISVPs and core particles[a]

Properties	Virions	ISVPs	Cores
Diameter (nm)[b]	80.3	72	57
Density in CsCl	1.36	1.38	1.43
Particle/ml per OD_{260} Unit	$2.1x10^{12}$	$2.7x10^{12}$	$4.2x10^{12}$
Outer capsid proteins			
$\sigma 1$	Present	Present	Absent
$\sigma 3$	Present	Absent	Absent
$\mu 1/\mu 1C$	Present	$\delta + \phi$[c]	Absent
Core proteins	Present	Present	Present
Spike protein $\lambda 2$	Present	Present	Present
Spike channel (8nm)[d]	No	No	Yes
10 dsRNA gene segments	Present	Present	Present
Infectious	Yes	Yes	No
Transcriptionally active	No	No	Yes

a: This table is modified from Nibert, Schiff and Fields (Nibert *et al.*, 1996).

b: The diameters of virions, ISVP and cores were measured by three different methods, negative-stain electron microscopy, cryoelectron microscopy and/or low-angle x-ray diffraction. These numbers presented in the table are the averages.

c: The $\mu 1/\mu 1C$ proteins are further cleaved to fragments of δ and ϕ when virions are converted to ISVPs (Pett *et al.*, 1973; Jayasuriya *et al.*, 1988).

d: Cryoelectron microscopy and computer reconstruction demonstrated these pentameric spike structures in core are significantly different from those in virions and ISVPs. A channel 8nm in diameter in each pentameric spike can be observed in cores, but not in virions and ISVPs (Dryden *et al.*, 1993). These channels are considered the pathways for extrusion of ssRNAs synthesized inside of cores.

structural proteins and three non-structural (NS) proteins encoded by each gene were determined by further electrophoretic studies (Loh and Shatkin, 1968; Smith *et al.*, 1969; Zweerink *et al.*, 1971; Cross and Fields, 1976; Ramig *et al.*, 1977). With the exception of the S1 gene which encodes both cell attachment protein σ1 and non-structural protein σ1s, the other nine genes are all monocistronic. This segmented genome allows reassortment during mixed infections with two different reovirus strains. The three mammalian reovirus serotypes are represented by prototype strains: T1Lang (T1L), T2Jones (T2J) and T3Dearing (T3D). The viruses were isolated from children whose names were respectively Lang, Jones, and Dearing (Sabin, 1959). These three serotype strains have almost identical structures, genome characteristics and complement fixing antigen, but they can be differentiated by the neutralization and hemagglutination inhibition assays, and most importantly, their dsRNA gene segments and proteins have different patterns and mobilities in polyacrylamide gels (Ramig *et al.*, 1977; Sharpe *et al.*, 1978) (Fig 2). The segmented nature of the viral genome and strain-dependent mobility differences of the genes give reovirus studies an unique tool, reassortant gene mapping. When cells are co-infected by two reovirus strains, the progeny viruses randomly encapsidate 10 gene segments from the two parents. Therefore, the ten gene segments of each progeny virus may be mixed (assorted); one or more genes are inherited

from one parent, while the remaining gene segments are from another parent. The genotypes of such resultant reassortant progeny viruses can be determined by resolving their isolated genomes by SDS-PAGE along with those of the two parents (Fig. 2). If there is a phenotypic difference between two reovirus strains, such as different amounts of viral yield in L cell cultures, the responsible gene(s) for this difference can be mapped by reassortant gene mapping. Since most of the genes are monocistronic, the responsible protein(s) can be determined as well (Fig. 2). For example, in Figure 2, the reassortant gene mapping method is performed based upon the following three aspects. First of all, reassortants A and B have the same phenotype as T1L, (i.e more viral yield in L cells) and have the L1 gene of T1L. Secondly, reassortants C and D behave like T3D and have L1 gene from T3D. Finally, all other 9 genes are randomly assorted. Then the L1 gene and its encoded minor core protein λ3 are responsible for this different phenotype seen between T1L and T3D. This method is extensively used in the Results section (3.6 and 3.7) of this thesis.

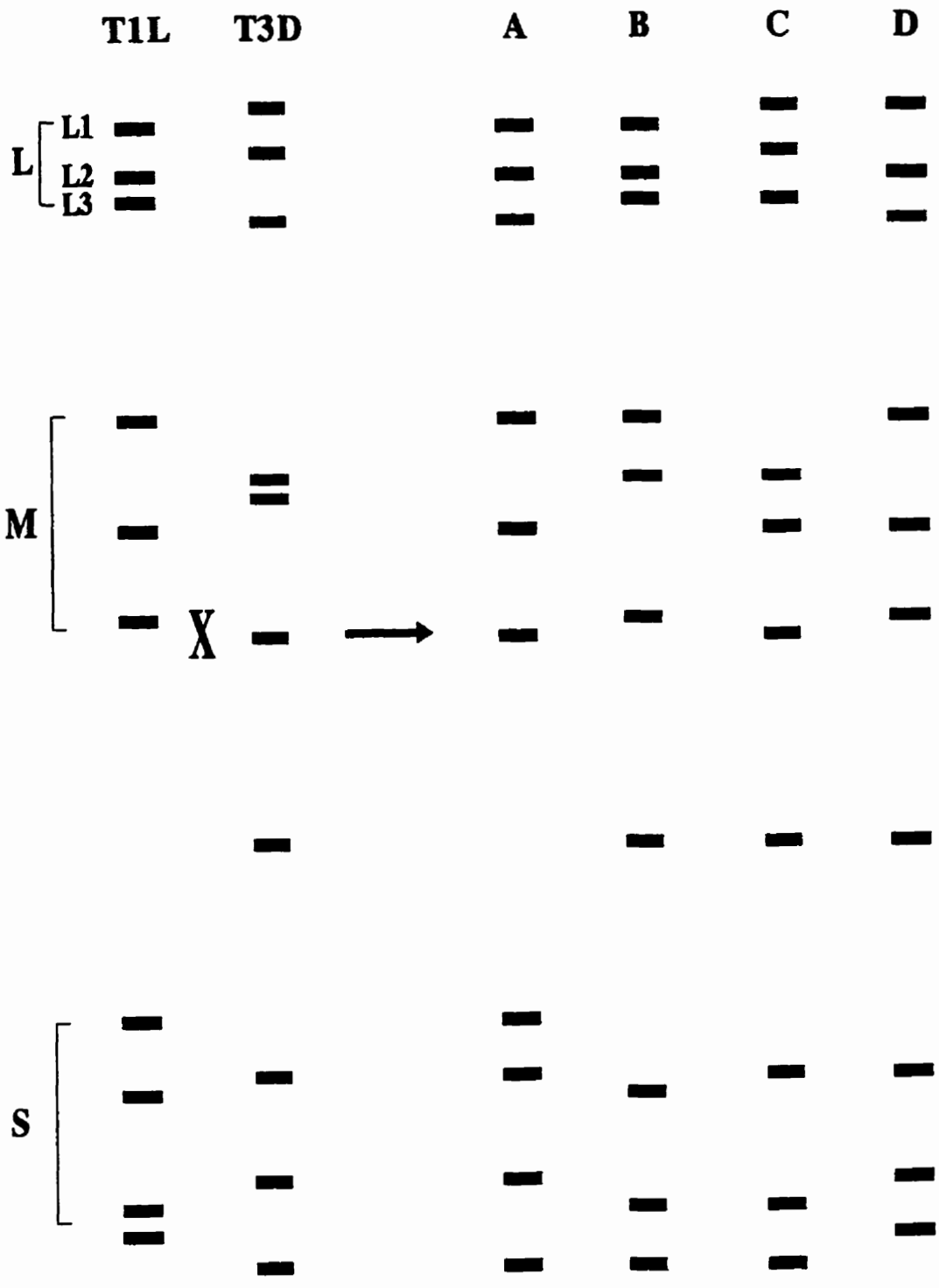

T1L
T3D
A
B
C
D
L
L1
L2
L3
M
X
S

Fig. 2. Reassortant gene mapping method. The mobilities of each of the 10 genes of T1L, T3D, and their reassortants by SDS-PAGE are shown in this cartoon. The orders of the 10 genes are L1, L2, L3, M2, M1, M3, S1, S2 for both T1L and T3D (from top to bottom), then S4 and S3 for T1L, but S3 and S4 for T3D. By comparing to the two parents, the genotypes of each reassortant can be deduced. In this example, the reassortant gene mapping method is performed based upon following three aspects. First of all, reassortants A and B have the same phenotype as T1L, (i.e more viral yield in L cells) and have the L1 gene of T1L. Secondly, reassortants C and D behave like T3D and have L1 gene from T3D. Finally, all other 9 genes are randomly assorted. Then the L1 gene and its encoded minor core protein λ3 are responsible for this different phenotype seen between T1L and T3D.

1.3. Reovirus replication cycle

In order to successfully replicate in cells, reovirus must attach to and penetrate the cells, uncoat to produce transcriptionally active cores, transcribe dsRNA genome to synthesize viral RNAs, translate mRNAs to produce viral proteins, assemble the progeny viruses and escape from cells as shown in Fig. 3. The first step in the replication cycle is entry. Reoviruses attach to cellular receptors (undefined) using cell attachment protein σ1, then penetrate into cells by receptor-mediated endocytosis (Borsa *et al.*, 1979, 1981; Sturzenbecker *et al.*, 1987; Rubin *et al.*, 1992). However, ISVP may penetrate into the cytoplasm directly through the plasma membrane (Sturzenbecker *et al.*, 1987; Lucia-Jandris *et al.*, 1993). After entry into the host cell, the next step (uncoating) occurs within endosomes and lysosomes where the outer shell proteins (σ3, σ1, μ1/μ1C) are cleaved. The uncoated products, the transcriptionally active cores are produced and released into cytoplasm by an unknown method. However, it is believed that low pH in those vesicles may facilitate this step (Canning and Fields, 1983). Unlike most viruses (poliovirus, HIV), the uncoating of reovirus is not complete (Silverstein and Schur, 1970; Silverstein *et al.*, 1970, 1972; Chang and Zweerink, 1971). Viral dsRNA genomes are always associated with core particles. This inner capsid (core) has a diameter of about 60nm. Protein λ2 is organized

as pentameric spikes which extend from the core capsid an additional 9.5nm at each icosahedral vertex (Luftig *et al.*, 1972; Ralph *et al.*, 1980). Cryoelectron microscopy and image reconstructions of active cores show that the spikes undergo significant conformational rearrangement during uncoating (Yeager *et al.*, 1997). Then, transcription occurs inside core particles. The minus strands of the 10 dsRNA gene segments are the only strands transcribed (Skehel and Joklik, 1969; Hay and Joklik, 1971). The newly-synthesized ssRNAs are extruded through the λ2 spike channels into the cytoplasm (Yeager *et al.*, 1997). Reoviruses synthesize two types of transcripts within infected cells, primary and secondary transcripts. The primary transcripts {capped at 5' end, $m^7G(5')pppN^{(m)}$} are produced by core particles derived from input particles (Chang and Zweerink, 1971; Silverstein *et al.*, 1972; Borsa *et al.*, 1981). The primary transcripts begin to appear 2 hours post-infection, reach a peak at 6 to 8 hours and become undetectable by 12 hours (Silverstein and Dales, 1968; Zweerink and Joklik, 1970; Joklik, 1972). Previous studies demonstrated that L1, M3, S3 and S4 (pre-early genes) are mainly produced during first several hours after infection (Zweerink *et al.*, 1972; Silverstein *et al.*, 1976). Then, the viral proteins encoded by these four pre-early genes are translated. The newly-made proteins may be important for production of the other six primary transcripts and later events in the replication cycle (Watanabe *et al.*, 1968;

Shatkin and LaFiandra, 1972; Lau *et al.*, 1975). For example, it has been shown that protein σ3 encoded by a pre-early gene S4 has dsRNA binding activity (Sharpe and Fields, 1982). In addition, σ3 can downregulate interferon-induced, dsRNA-activated protein kinase (Imani and Jacobs, 1988; Lloyd and Shatkin, 1992). These studies suggest that protein σ3 may play a role in the inhibition of cellular RNA and protein synthesis in reovirus infected cells. Also, non-structural proteins μNS and σNS encoded by other two pre-early genes M3 and S3 may assort and condense the 10 gene segments, then pack them into mRNA assortment complex formed mainly by λ1 and σ2 (Stamatos and Gomatos, 1982; Antczak and Joklik, 1992; Xu *et al.*, 1993). More proteins (possibly λ3 and μ2 ?) are incorporated into capsid structure (transcriptase complex) where minus stranded RNAs are synthesized and associate with their plus ssRNA templates. Then, the secondary uncapped transcripts are produced by newly-assembled particles (RNase-resistant transcriptase particles). These secondary transcripts become detectable by 4 to 6 hours after infection, reach a maximum at 12 hours, then subsequently decrease (Kudo and Graham, 1966; Watanabe *et al.*, 1967, 1968; Skup *et al.*, 1981). Secondary transcripts are the major source for viral proteins and minus stranded RNA synthesis in reovirus infected cells (Ito and Joklik, 1972a; Zarbl and Millward, 1983). It has been proposed that protein σ3 may enhance the translation of viral uncapped mRNA by either inhibiting capped cellular

mRNA synthesis or inducing viral translation in a localized area (Skup and Millward, 1980; Skup *et al.*, 1981; Lemieux *et al.*, 1984). The last step of replication is assembly of the viral capsids and release from cells (Fig. 3).

1.4. Enzymatic functions associated with core particles

Previous studies demonstrated that production of viral RNA in L cells was not inhibited by actinomycin D at a concentration of 0.5μg/ml (Kudo and Graham, 1965; Shatkin, 1965). At this concentration, approximately 90% of the cellular RNA synthesis is halted, suggesting the cellular enzymes are not involved in reovirus transcription. Furthermore, *in vitro*, the core synthesizes copies of all ten segments of viral RNA and the resulting 10 single-stranded mRNA molecules are the same as transcripts synthesized *in vivo* (Chang and Zweerink, 1971; Hay and Joklik, 1971; Shatkin, 1974; Furuichi *et al.*, 1975). Therefore, the core is a multiple enzyme complex that contains all necessary components for transcription, methylation, and capping of progeny mRNA, including polymerase, helicase, RNA triphosphatase, guanylyltransferase and methyltransferase. Most of these enzymatic activities have not yet been assigned to core proteins. Little is known about details of core structure and how this particle transcribes mRNA from the enclosed genomic RNA. Structural studies indicated that each virion possesses 120 copies of each major core proteins λ1 and σ2 (Matsuhisa

Release
Assembly
Transcriptase complex
mRNA assortment complex
Uncapped secondary mRNAs
Viral proteins
10 capped primary mRNAS
low pH?
endosomes/ lysosomes
NUCLENS
Penetration
ISVP
Receptor mediated endocytosis
Virion Attachment

Fig. 3. Reovirus replication cycle. Reoviruses attach to and enter cells by receptor-mediated endocytosis. However, ISVPs may penetrate into the cytoplasm directly through the plasma membrane. After entry into the host cell, the next step (uncoating) occurs within endosomes and lysosomes where the outer shell proteins are cleaved. The uncoated products, cores are produced and released into cytoplasm. Then, transcription occurs inside core particles. The newly-synthesized ssRNAs are extruded through the λ2 spike channels into the cytoplasm. The primary transcripts (capped at 5' end), including the four pre-early genes L1, M3, S3 and S4 are produced by core particles derived from input particles. Then, viral proteins are made and the transcriptase complexes are assembled. Within these structures, the minus stranded RNAs are replicated and the secondary uncapped transcripts are produced. More viral proteins are synthesized from the uncapped mRNAs. The last step of replication is assembly of the viral capsids and release from cells. lines (_____) represent viral ssRNA. ■ is the symbol of 5' cap structure on viral ssRNA. Mark (?) suggests uncertain step. Since reovirus replication is a cytoplasmic event, the cell nucleus is deliberately minimized.

and Joklik, 1974; White and Zweerink, 1976; Xu *et al.*, 1993). The large copy numbers of these two proteins suggest λ1 and σ2 may function mainly in building the architectural framework of the core. By contrast, each core particle has 12 pentameric spikes (= 60 copies) and 12 copies of each minor core proteins λ3 and μ2 (Luftig *et al.*, 1972; White and Zweerink, 1976; Ralph *et al.*, 1980). The small copy numbers of the minor proteins λ3 and μ2 imply that they are more likely involved in enzymatic functions. Nevertheless, proteins λ1 and λ2 have been labelled with pyridoxal phosphate, a known affinity reagent for cellular and viral nucleic acid polymerases, suggesting possible roles for them in RNA synthesis. Major core proteins λ1 and σ2 may be important for the positioning or movement of dsRNA templates during transcription, due to their dsRNA-binding activities (Schiff *et al.*, 1988; Dermody *et al.*, 1991). In addition, reassortant gene mapping analysis indicated that major protein λ1 has ATPase activity related to transcription (Noble and Nibert, 1997a). Futhermore, recombinant study showed that expressed λ1 protein in yeast has helicase activity (Bisaillon *et al.*, 1997). Biochemical studies showed λ2 proteins could form covalent λ2-GMP complexes when cores were incubated with [α-^{32}P]GTP, suggesting the λ2 spikes possess guanylyltransferase activity (Shatkin *et al.*, 1983; Cleveland *et al.*, 1986). Most importantly, vaccinia-expressed λ2 has all the enzymatic activities expected of the guanylyltransferase (Mao and Joklik, 1991).

Protein λ2 may also methylate the 5' end of reovirus mRNAs (Seliger *et al.*, 1987; Koonin, 1993). Previous studies demonstrated that the transcriptase of reovirus serotypes T2J and T3D had different pH optimum. Reassortant gene mapping studies determined that minor core protein λ3 (encoded by the L1 gene) was responsible for this phenotypic difference (Drayna and Fields, 1982). In addition, regions of protein λ3 share sequence homology including the GDD sequence motif with some other RNA polymerases (Morozov, 1989; Bruenn, 1991). Most convincing evidence came from recombinant studies which showed recombinant λ3 has poly(C)-dependent poly(G) polymerase activity (Starnes and Joklik, 1993). The above studies strongly suggest that minor core protein λ3 is the RNA dependent RNA polymerase (RDRP). However, expressed λ3 alone lacks specificity and processivity (Starnes and Joklik, 1993), implying ancillary proteins are required for accurate and efficient genomic transcription.

The purpose of the research detailed in this thesis was to better characterize the reovirus RNA dependent RNA polymerase. This study showed that the M1 gene, which encodes minor core protein μ2, was uniquely associated with the difference in temperature optimum of transcription. The L1 gene, which encodes minor core protein λ3, previously determined as the reovirus RNA dependent RNA polymerase, and the M1 gene were associated with the difference in absolute amount of transcript produced. These results suggest that

minor core protein μ2 also plays a role in reovirus transcription and for the first time clearly identified that minor core protein μ2 is the co-factor required by protein λ3 for its accurate RNA dependent RNA polymerase activity (Yin *et al.*, 1996). We also found that the cores treated with 65^{0}C and protease digestion lost their spike structures and λ2 proteins but not genomes and that these spike-deficient cores showed functional transcriptase activity. All prior attempts to partially disassemble the reovirus core structure completely destroyed its enzymatic activity (White and Zweerink, 1976; Cashdollar, 1994). Therefore, this is the first identification of transcriptase activities in partially disassembled cores (Yin *et al.*, 1997).

Collectively, this study has further defined the roles of core proteins in reovirus transcription. The minor core proteins λ3 and μ2 appear to associate and form the reovirus RNA dependant RNA polymerase complex which produces ssRNAs from genomic dsRNA. The pentameric core spike structure and intact protein λ2 are not required for reovirus transcriptase activity, but may only be involved in modification of the newly-synthesized mRNA (capping).

2. MATERIALS AND METHODS

2.1. Cells and viruses. Reovirus type 1 Lang (T1L), type 3 Dearing (T3D), and T1L x T3D reassortant viruses [originally isolated as described (Drayna & Fields, 1982b; Brown *et al.*, 1983; Coombs *et al.*, 1990)] are laboratory stocks that were grown in mouse L929 cell monolayers in Joklik modified minimal essential medium (MEM) (GIBCO, Grand Island, NY) supplemented to contain 2.5% fetal calf serum (Intergen, Purchase, NY), 2.5% VSP neonate bovine serum (Biocell, Carson, CA), 2*mM* glutamine, 100U penicillin per *ml*, 100μg streptomycin sulfate per *ml*, and 1μg amphotericin-B (GIBCO, Grand Island, NY) per *ml*, essentially as previously described (Coombs *et al.*, 1994). L929 cells are maintained in suspension culture at $4.5x10^5$/*ml*. Cells are counted daily using the light microscope. Based upon cell concentration, fresh MEM is exchanged to keep cells in optimum condition ($4.5x10^5$/*ml*).

2.2. Plaque assay and virus passaging. Virus stocks including wild types and reassortants were plaque purified and amplified through two passages in supplemented Joklik's modified minimal essential medium (MEM) at 37^0C. Mouse L929 cells (1.1 x 10^6 cells/well) were plated onto Corning Costar 6-well dishes (Fisher, Nepean, Ontario) to form monolayers. On the next day, 0.1*ml* of each of 6 serial 1:10 dilutions of the virus stocks in gel saline were added onto cell

monolayers. Adsorption of the innoculums were carried out at room temperature for 1 hr. After adsorption, infected cells were overlayed with 3*ml*/well with a 50:50 ratio of 2% agar and 2 x Medium 199 supplemented (completed) with 2.5% fetal calf serum, 2.5% VSP, 2*mM* glutamine, 100U penicillin per *ml*, 100μg streptomycin sulfate per *ml*, and 1μg amphotericin-B per *ml*, then incubated at 37^{0}C. Cells were fed with 2*ml* fresh agar/Medium 199 after 3 days of infection and stained with 0.04% Neutral Red solution about one week after infection. The plaques were counted and viral titers were calculated. Duplicate dilutions in one 6-well plate or multiple plates for one clone were used for more accuracy. The selected plaques were picked using sterile pasteur pipets (Fisher, Nepean, Ontario) and suspended in 1 *ml* gel saline as plaque purified virus stocks (P_0). Subconfluent monolayers (~90-95%) in Corning T_{25} flasks were infected with 1 *ml* P_0 stocks. Adsorption of viruses were carried at room temperature for 1 hr, then infected cells were incubated at 37^{0}C after being overlaid with 5*ml*/flask completed MEM. Cells were examined daily with the light microscope to check for cytopathic effect (CPE). When monolayers showed 80-90% CPE, infected cells were frozen in a -80^{0}C freezer. The viruses in cell aliquots (P_1) were collected after freeze-thawing 3 times. Aliquots of 0.3 *ml*/Corning T_{75} flask were used to amplify more viruses (P_2) in a similar manner. The dsRNA genomes of these viruses were extracted and the virus stock types were confirmed in SDS-PAGE

as described below.

2.3. Cytoplasmic extraction of reovirus dsRNA genome. L929 cell monolayers in Corning P_{60} culture dishes (Fisher, Nepean, Ontario) were infected with viral P_2 stocks and incubated at 37^{0}C as described above. After showing 80-90% CPE, infected cells were transfered to centrifuge tubes and pelleted by centrifugation at 500Xg for 10 min. The cell pellets were resuspended in 450 μl NP-40 buffer (140mM NaCl, 1.5mM $MgCl_2$, 10mM Tris, pH 7.4) and dissolved by adding 50 μl 5% NP-40, then incubated for 30 min on ice. Cellular nuclei and organelles were spun down as described above and supernatants were removed to microfuge tubes (Fisher, Nepean, Ontario). The viral dsRNA genomes were purified by Phenol/Chloroform extraction and precipitated by 3M NaOAc and ice-cold absolute EtOH. The precipitated dsRNA genomes were pelleted in Biofuge A (VWR Scientific of Canada Ltd, Toronto, Ontario) at 12,500 rpm for 30 minutes. The dsRNA pellets were lyophilized for 30 minutes, then resuspended in electrophoresis sample buffer and resolved by 10% SDS-PAGE as described below.

2.4. Purifications of reovirus particles. For each purification, a total of 6.5 x 10^8 cells in a suspension culture were centrifuged at 700 rpm for 11 minutes, and the pellet was re-suspended in MEM containing 2.5% FCS, 2.5% VSP, 100U Penicillin per ml, 100μg streptomycin sulfate per ml, and

1μg amphotericin-B per *ml*. A second-passage reovirus stock was added at a MOI (multiplicity of infection) of 5 PFU/cell and allowed to adsorb for 1 hour at 37^{0}C. The infected cells were diluted to a concentration of 6.5 x 10^5 cells per *ml* with 75% of fresh MEM completed with FCS, VSP, Penicillin, streptomycin sulfate, and amphotericin-B described above, and 25% pre-adapted medium from cell suspension. The infected cells were incubated at 33.5^{0}C, for 65 hours, then centrifuged at 1,100rpm for 15 minutes. The cell pellet was resuspended in 10 *ml* HO buffer (Homogenization buffer, 10 *mM* Tris, pH 7.4, 250 *mM* NaCl, 10 *mM* 2-mercaptoethanol), and frozen at -80^{0}C for at least 30 minutes, then thawed. The sample was sonicated for about 10s to break up the clumps that formed during freeze-thaw. To break cell membranes, 1/50th volume of 10% DOC (deoxycholate) was added and incubated on ice for at least 30 minutes. Then, 1/2 volume of Freon 113 (1,1,2-Trichloro-1,2,2-Trifluoroethane) (Caledon Laboratories LTD, Georgetown, Ontario) was added into suspension and made an emulsion by sonicating for several seconds at 50% duty cycle with the sonicator probe (Vibra Cell) (Sonics & Materials Inc, Danbury, CN). Another 1/2 volume of Freon was added and solution re-emulsified. The mixture was centrifuge at 5000rpm for 10 minutes in a fixed-angle rotor (JA-20) in Beckman RC centrifuge (Beckman, Mississauga, Ontario) to separate the inorganic/organic phases. The top phase was pipetted into a fresh tube and re-extracted with Freon as described above.

The resulting twice-extracted aqueous phase was layered directly onto a preformed CsCl density gradient(1.22-1.44 g/*ml*), and centrifuged in a Beckman SW28 rotor at 25,000rpm for 6 hours. The purified virus particle band was collected and dialysed against 1 X SSC(150 *mM* NaCl, 15*mM* Sodium Citrate, pH 7). The concentration of purified viruses was measured (with the relationship 1 ODU_{260} = 2.1 × 10^{12} particles per millilitre) as described before (Smith *et al.*, 1969).

2.5. Digestion and purification of core particles. Previous studies indicated that the concentrations of virus is critical for the conversion of reovirus virion to core (Drayna and Fields, 1982). It was also found that different serotypes require different optimum virus concentrations for core production, ≥5 x 10^{13}/*ml* for T1L and ≥5 × 10^{12}/*ml* for T3D. In addition, the M2 gene (encoding outer capsid protein μ1/μ1C) is responsible for this phenotypic variation between T1L and T3D (Drayna and Fields, 1982). Therefore, for T1L and reassortants with the M2 gene of T1L, purified viruses were adjusted to a concentration of 6.5 × 10^{13}/*ml* with 1 X SSC. By contrast, the virus concentrations of T3D and reassortants with the M2 gene of T3D were set to 2.5 × 10^{13}/*ml*. Then, these viruses were digested with 100 μg/*ml* chymotrypsin at 37°C for 3 hours. At the end of the incubation, the mixtures were loaded onto 1.22-1.5 g/*ml* CsCl density gradients and centrifuged at 30,000 rpm for 6 hours in a Beckman SW41 rotor.

Core particle bands were collected and dialysed against core buffer (1 M NaCl, 100 *mM* $MgCl_2$, 25 *mM* HEPES pH 8.0). The concentration of purified cores was measured, using the relationship $1OD_{260}$ = 4.2 x 10^{12} particles per millilitre, glycerol was added to a final concentration of 25%, and core aliquots frozen at -80°C. This treatment had no detectable effect upon core structure or function compared to non-treated, non-frozen cores. In some experiments, cores were preheated at various temperatures ranging from 62°C to 70°C for various length of times, then chilled in an ice/water bath, immediately before some of the following assays. Core purity was monitored by characteristic protein profiles in SDS polyacrylamide gels and by electron microscopy (for example, see Fig. 1). The gene patterns of all reassortants used were verified by electropherotyping as described (Sharpe *et al.*, 1978; Hazelton & Coombs, 1995).

2.6. Western Blot analysis. Samples were dissolved in electrophoresis sample buffer (0.24 M Tris, pH 6.8, 1.5% dithiothreitol, 1% SDS), then heated at 95°C for 5 min, and resolved in 5-15% SDS-PAGE (16.0 × 12.0 × 0.1 cm) at 20mA for 6 hr. Then, the gels were collected and soaked in TransBlot buffer (48*mM* Tris, 39*mM* Glycine, 0.0375% SDS and 20% MeOH) for 20 minutes. At the same time, for each gel, two pieces of filter paper and one membrane (Immobilon-P Transfer Membranes) (Millipore, Bedford, MA) were also soaked in TransBlot buffer

for 20 minutes. Each transferring sandwich consisted of the following order: (from bottom to top) one filter paper, membrane, gel and another filter paper was placed onto a BIO-RAD transfer cell (TRANS-BLOT SD, SEMI-DRY TRANSFER CELL, BIO-RAD). The transfer of proteins from SDS-PAGE to membranes was performed at 15V for 30 minutes. After transfer, membranes were washed with dH_2O, then stained with 2% Ponceau S {3-hydroxy-4-[2-sulfo-4-(4-sulfophenylazo)-phenylazo]-2,7-naphthalenedisulfonic acid, Sigma, Oakville, Ontario}. Membranes were rinsed with dH_2O three times until protein bands became visible, then soaked in Blocking Buffer [5% Skim milk in TBST Buffer (20 mM Tris-base, 100 mM NaCl and 0.05% Tween 20) and 0.04% Sodium Azide] for 2.5 hours. Various rabbit antisera (anti-μ1, anti-σ3 or anti-whole virus) were used to incubate different membranes for 90 minutes. Membranes were then washed with TBST to remove non-specifically bound primary antibodies. Then, secondary antibody (1:1,500), anti-rabbit IgG coupled with Horse Radish Peroxidase (HRP) were added into reactions and incubated for 90 minutes. The membranes were washed with TBST four times. Then, the bands were developed by treating membranes with staining solution [0.5% 3.3'-diaminobenzidine (DAB), 0.16% CoCl2 and 1% H_2O_2, dissolved in phosphate-buffered saline (PBS)]. When the bands were of the desired intensity, membranes were washed with dH2O, then dried between multiple layers of absorbent paper.

2.7. *in vitro* transcriptase assay. Aliquots of cores were thawed and diluted into Core Buffer, diethylpyrocarbonate-treated distilled H_2O (DEPC dH_2O), and reaction buffer such that each 50 μl transcriptase assay reaction contained 3.5×10^{11} gradient purified core particles; 2*mM* each of ATP, CTP, GTP, and UTP; 3.3*mM* phosphoenol pyuvate; 100ng/μl pyruvate kinase; 0.8 units/μl RNAsin (Boehringer Mannheim, Laval, Québec); 9.5*mM* $MgCl_2$; and 100*mM* Hepes, pH 8.0. To reduce pipeting errors a single reaction mixture was prepared for each type of core tested and divided into aliquots. The reactions were labelled by adding 0.1μCi ^{32}P-UTP (New England Nuclear, Missisauga, Ontario, Canada), and incubated at different temperatures for 1 h. Reactions were stopped by chilling on ice, then transcripts were precipitated with 8 volumes of ice-cold 5% Trichloroacetic Acid (TCA), collected onto filters, washed with 5% TCA and ethanol. Radioactivities representing the amounts of synthesized ssRNAs by cores at different temperatures were measured in a Beckman LS 5000CE scintillation spectrophotometer.

2.8. Statistical analyses. The relative contribution of any single gene in reassortant mapping experiments was determined by both nonparametric and parametric methods to ensure that conclusions derived were independent of the statistical method used. The nonparametric Wilcoxon rank sum test (Hassard, 1991) was performed by ranking transcriptase temperature

optimum ratios or transcriptase efficiencies obtained from different reassortant virus cores. Parametric regression analyses used transcriptase temperature optimum ratios or transcriptase efficiencies as the dependent variable predicted by parental origin of the 10 reovirus gene segments. The proportion of variance (R^2) for the phenotypic response predicted by the parental origin of each gene segment, as well as various sets of genes, were determined. All statistical analyses were performed on all individual experimental values as well as on averages obtained from multiple experiments. All analyses were conducted with the SAS (SAS/STAT Users Guide, 1990).

2.9. Electron microscopy(EM) of reovirus core particles. Standard drop method preparations of treated core particles were prepared and negatively stained with 12*mM* Phosphotungstic Acid as described (Hammond *et al.*, 1981). Briefly, a drop of each core sample was placed on clean parafilm. Then, carbon-coated 400-mesh electron microscope copper grid (3.05mm) was turned over onto the surface of the sample drop for 30 seconds to 1 minute. The excess sample was drawn off by gently touching a torn edge of a piece of filter paper to an edge of the grid. The sample on the grid was stained with 1.6% Phosphotungstic Acid, pH 7 for 30 seconds to 1 minute. The excess stain was also drawn off. Samples were then viewed using a Philips model 201 electron microscope at

magnifications between 30,000x and 100,000x. Three to five preparations were evaluated for each treatment, and relative proportions of particle types in each preparation were determined from at least five randomly selected and well separated grid squares. The electron micrographs were recorded on Kodak 5302 direct positive film and printed on Kodak Polycontrast III paper.

2.10. Hybridization of ssRNA made *in vitro* with cold dsRNA genome. Methods of hybridization assay were modified from Ito (Ito and Joklik, 1972b). *In vitro* transcriptase assays were performed with[^{32}P]UTP at different temperatures as described above. After 1 hr incubation, core particles were pelleted (Beckman airfuge ® 26psi, 5 min). Labelled single stranded RNAs in 50μl aliquots of the supernatants were supplemented with 10 μg Yeast tRNA and rRNA (carrier RNA to facilitate precipitation) and precipitated by the addition of 1/10 volumes of NaOAC and 2.5 volumes of ice-cold absolute EtOH at -20^{0}C overnight. The precipitated ssRNAs were pelleted by microcentrifugation at 12,800 rpm for 30 min, then resuspended in 68μl annealing buffer 1 (90% DMSO, 10% Tris-EDTA buffer and 20 units of RNasin). 2 μl of 40ng/μl of unlabelled T1L dsRNA genomes were added and the mixtures were incubated at 50^{0}C for 45 min, then 30μl of annealing buffer 2 (100 *mM* NaCl, 10 *mM* Tris, pH 7.4, preheated to 50^{0}C) was added to each reaction. Hybridizations were performed in a programmable thermal cycler

by allowing the reaction mixture temperature to decline from 50°C to 37°C at a rate of 0.1°C per minute, and completed by an overnight 37°C incubation. Hybridization products were precipitated by 3M NaOAC and absolute EtOH at -20°C overnight, and pelleted as described above. Pellets were washed with 80% EtOH, then re-pelleted. The pellets were dried in a Speed Vac (SC100) for 20 min then suspended in 80μl of 2 X SSC. RNase A was added to a final concentration of 12.5 $\mu g/ml$ and the mixtures incubated at 37°C for 15 min to digest unhybridized ssRNA. The hybridization products were purified by Phenol/Chloroform extraction, then resuspended in electrophoresis sample buffer and resolved in 10% SDS-PAGE as described below.

2.11. ***In vitro*** **translation assay.** *In vitro* transcriptase assays were performed at different temperatures as described above to make ssRNAs except that[^{32}P]UTP was omitted. After 1 hour incubation, transcriptase reactions were stopped and core particles were pelleted as above. 5μl aliquots of supernatants were added into 15 μl of the Methionine-deficient nuclease treated Rabbit Reticulocyte Lysate System (Promega, Madison, Wis). Reactions were supplemented with 10 units RNasin, labelled with 6.25 μCi ^{35}S-Methionine (ICN Biomedical, Costa Mesa, CA), then incubated at 30°C for 1 h. Viral proteins were immunoprecipitated with anti-T3D reovirus-conjugated Protein A Sepharose (SPA) beads (Pharmacia,

Uppsala, Sweden) and resolved in 5-15% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) as described (Hazelton and Coombs, 1995).

2.12. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). All samples to be analyzed by SDS-PAGE were dissolved in electrophoresis sample buffer (0.24 M Tris, pH 6.8, 1.5% dithiothreitol, 1% SDS). RNA samples were heated to 65^{0}C for 5 min, and resolved in 10% SDS-PAGE (16.0 × 16.0 × 0.15 cm) at 18mA for 43 hr. Non-labelled RNA samples were stained by Ethidium Bromide and photographed with Polaroid 667 film. [^{32}P] labelled RNA samples were resolved similarly, then gels were fixed and autoradiographed.

Protein samples were heated to 95^{0}C for 5 min and resolved in either 5-15% SDS-PAGE (Laemmli, 1970) (16.0 × 12.0 × 0.1 cm) at 20mA for 6 hr or in hybrid tris-glycine-urea (TGU) gels (16.0 × 16.0 × 0.1 cm) at 8 mA for 15 hr (Coombs, 1997). The TGU gel is a Laemmli-type gel supplemented with 7M Urea which can resolve all the reovirus structural proteins and shows T1L/T3D strain-dependant electrophoretic mobility polymorphisms for most of the proteins. This hybrid resolving gel consisted of a 4cm step of 4% SDS-PAGE (using the tris-glycine (Laemmli) system which contains no urea) placed on top of a 14cm tall 4-16% exponential gradient SDS-Tris-Glycine-PAGE that contained 7M urea. The identities of λ2 and other viral proteins in the gel were proved by gene mapping using

T1L, T3D, and their reassortants, and by peptide digestion assay (Coombs, 1997).

Proteins and RNA were detected by silver-staining (Merril *et al.*, 1981), unlabelled proteins were visualized by Coomassie Brilliant Blue staining, and radiolabelled viral peptides were detected by fluorography (Kodak X-AR X-ray film, Rochester, NY) (Hames and Rickwood, 1981). Gels were scanned with a LKB Ultroscan XL laser densitometer. Band intensities were analyzed in the Scanplot program as described (Jiang *et al.*, 1991).

2.13. Sequence determination of the T2J M1 gene.

2.13.1. Synthesis of M1-specific primers for T1L and T2J M1 gene sequencing. The nucleotide sequence of the T1L M1 gene has been determined (Zou and Brown, 1992)(see section 4.2, Fig. 23). In addition, previous studies showed that the three serotypes of reovirus have very high homology (~90%) in nine of ten genes, except the S1 gene (Nibert *et al.*, 1996). Therefore, two primers complementary to both ends of T1L M1 gene were designed and used in the sequencing of both T1L and T2J M1 gene. One primer (M1-01) (5' CGATAAGCGCCAGTACCG 3') was complementary to 3' end of negative strand. Another primer (M1-07) (5' CTTCGCGCATGCATCAG 3') was complementary to 3' end of plus strand. The primers were synthesized in a Beckman oligo 1000 DNA synthesizer.

2.13.2. Reverse transcriptase polymerase chain reaction (RT-PCR). The genomic dsRNA of T1L and T2J were Phenol/Chloroform extracted as described in Section 2.3, except one extra phenol/chloroform extraction and one chloroform only extraction were used to isolate pure dsRNA. An aliquot of 6 μl purified T1L or T2J dsRNA in 90% DMSO (Dimethyl sulfoxide) and 10% 10*mM* Tris, pH 6.8 were placed into sterile 0.6*ml* RNase-free microcentrifuge tube (Fisher, Nepean, Ontario) and heated at 50^{0}C for 45 minutes to melt dsRNA. Samples were then transferred to an ice/water bath to snap-cool dsRNA and 63μl ice-cold DEPC-dH_2O was immediately added. The other components for first stand cDNA synthesis were added, including 32 Units RNasin (Boehringer), 0.1 μM each oligonucleotide primer (M1-01 and M1-07 see Section 2.13.1), 0.5*mM* dNTP mix (dATP, dCTP, dGTP, and dTTP), 20 μl 5 x 1st Strand Buffer (250*mM* Tris-HCl, pH 8.3, 375*mM* KCL, 15*mM* $MgCl_2$) (GIBCOBRL), 0.001% BSA, 1*mM* DTT (dithiothreitol), 1.6 Unit Superscript enzyme (GIBCOBRL) in total of 100μl reaction. These reactions were incubated at 42.5^{0}C for 1 hour.

The first strand products were then amplified by PCR. Each amplification reaction (100 μl) contained 16μl of the first stand cDNA, 69μl sterile dH_2O, 1μM each oligonucleotide primer (M1-01 and M1-07 as described in Section 2.13.1), 1 x Taq polymerase Reaction Buffer (20*mM* Tris-HCl, pH 8.4), 0.5*mM* dNTP mix, 2*mM* $MgCl_2$, 0.025 Unit Taq polymerase (Perkin-Elmer Ampli-taq^{tm}). The samples were covered with 45 μl PCR Oil and

placed into Preprogrammed Thermal Cycler (PTC-100) (MJ Research INC, Watertown, Mas). The amplifications were performed under the following conditions: 5 cycles of 94^{0}C for 3 minutes, 45^{0}C for 1.5 minutes, 72^{0}C for 3 minutes; followed by 31 cycles of 92^{0}C for 1.5 minutes, 45^{0}C for 1.5 minutes, 72^{0}C for 3 minutes; and a final elongation step of 10' at 72^{0}C, then cooling to 4^{0}C. The amplified cDNAs were screened in 1% agarose gel and purified using Bio-Rad Prep-A-Gene kit (Bio-Rid, Hercules, CA). Briefly, the cDNAs were dissolved in 1% agrose gel. The bands at 2.3 kb (the size of the M1 gene) were excised and treated with Prep-A-Gene binding buffer (3 x volumes of the gel) at 42^{0}C for 5 minutes. The Prep-A-Gene matrix (5 $\mu l/\mu g$ DNA) was added to bind the DNA molecules at room temperature for 10 minutes, then pelleted in Biofuge A (VWR) at 15,000 rpm for 30 seconds. The pellets were rinsed with binding buffer and re-centrifuged as above. The pellets were washed twice with Prep-A-Gene wash buffer (2.5 x volumes of the gel) and spun down as above. The DNAs bound to matrix were eluted by Prep-A-Gene elution buffer (1.5 x volumes of the pellet) and stored at -20^{0}C. The size and concentration of the isolated DNA were determined in 1% agarose gel.

2.13.3. End-labelling cycle sequencing of the T1L and T2J M1 gene. The system was described in Gibco instruction book (BRL # 8196S) and was originally referenced by Murray and Craxton (Murray, 1989; Craxton, 1991). The primers, M1-01 and

M1-07 (see Section 2.13.1 were end-labelled with [γ^{32}P]-ATP. Each end-labelling reaction (5 μl) contained 0.2 μM primer, 1x Kinase Buffer (5mM, Tis-HCl, 1mM $MgCl_2$ and 0.5mM DTT, pH 7.5) and 1.1 units T4 Polynucleotide Kinase (Boehringer). Each reaction was labelled with 6.4 μCi [γ-^{32}P]ATP and incubated at 37°C for 10 minutes. The kinase was destroyed by incubation at 55°C for 5 minutes. Then, each end-labelled primer was added to a aliquot of 31 μl reagents including 1x Taq Buffer, 5mM $MgCl_2$, 69ng (30ng/kb) T1L or T2J cDNA (see Section 2.13.2) and 0.6 units Taq polymerase (Gibco). An aliquot of 8 μl/each above reaction was mixed with 2 μl/tube of each ddNTP solution and marked as A, C, G, T in different coloured tubes. The sequencing reactions were covered with 20μl PCR Oil and performed in Programable Thermal Cycler under following conditions: 95^{0}C for 3 minutes; followed 19 cycles of 95^{0}C for 30 seconds, 55^{0}C for 30 seconds, 70^{0}C for 1 minutes; then 9 cycles of 95^{0}C for 1.5 minutes, 70^{0}C for 1 minutes; finally cooling to 4^{0}C. The sequencing reactions were terminated by adding 5μl of Stop Solution (20mM EDTA, 0.04% bromophenol blue and 0.04% xylene cyanol) and stored at -20°C. The sequencing products (A, C, G, T) were analysed in 6% sequencing gels at 1500V. Then, gels were fixed and autoradiographed.

3. RESULTS

3.1. Genotypes of reovirus wild-type T1L, T3D and their reassortants are determined from 10% SDS-PAGE. To perform reassortant gene mapping, reovirus serotypes T1L, T3D and T1L x T3D inter-typic reassortants were plaque-purified and grown through two passages in L929 cells. The dsRNA genomes of these clones were then phenol/chloroform extracted as described in Section 2.3. The purified RNAs were heated to 65^{0}C and resolved by SDS-PAGE. The genotypes of these reassortants were deduced based upon their mobility patterns in SDS-PAGE. Some of the reassortants used in this study are shown in Fig. 4 and Table 2. For example, in reassortant EB47, the uppermost gene (L1) migrates the same as does the L1 gene in T1L. The next gene (L2) of EB47 has the same mobility as does the L2 gene of T3D. The other eight genes of EB47 all migrate the same as do those of T1L. Therefore, the genotype of this reassortant, EB47 is: 1, 3, 1, 1, 1, 1, 1, 1, 1, 1 as shown in Table 2. Reassortants used in this study and their gel-deduced electropherotypes are shown in Table 2, 3 and 5.

3.2. Optimum conditions of reovirus core production. Reovirus core particles can be generated *in vitro* by digesting virions with the protease chymotrypsin. The digestion conditions were determined based upon a previous study (Drayna and Fields, 1982). After CsCl gradient purification,

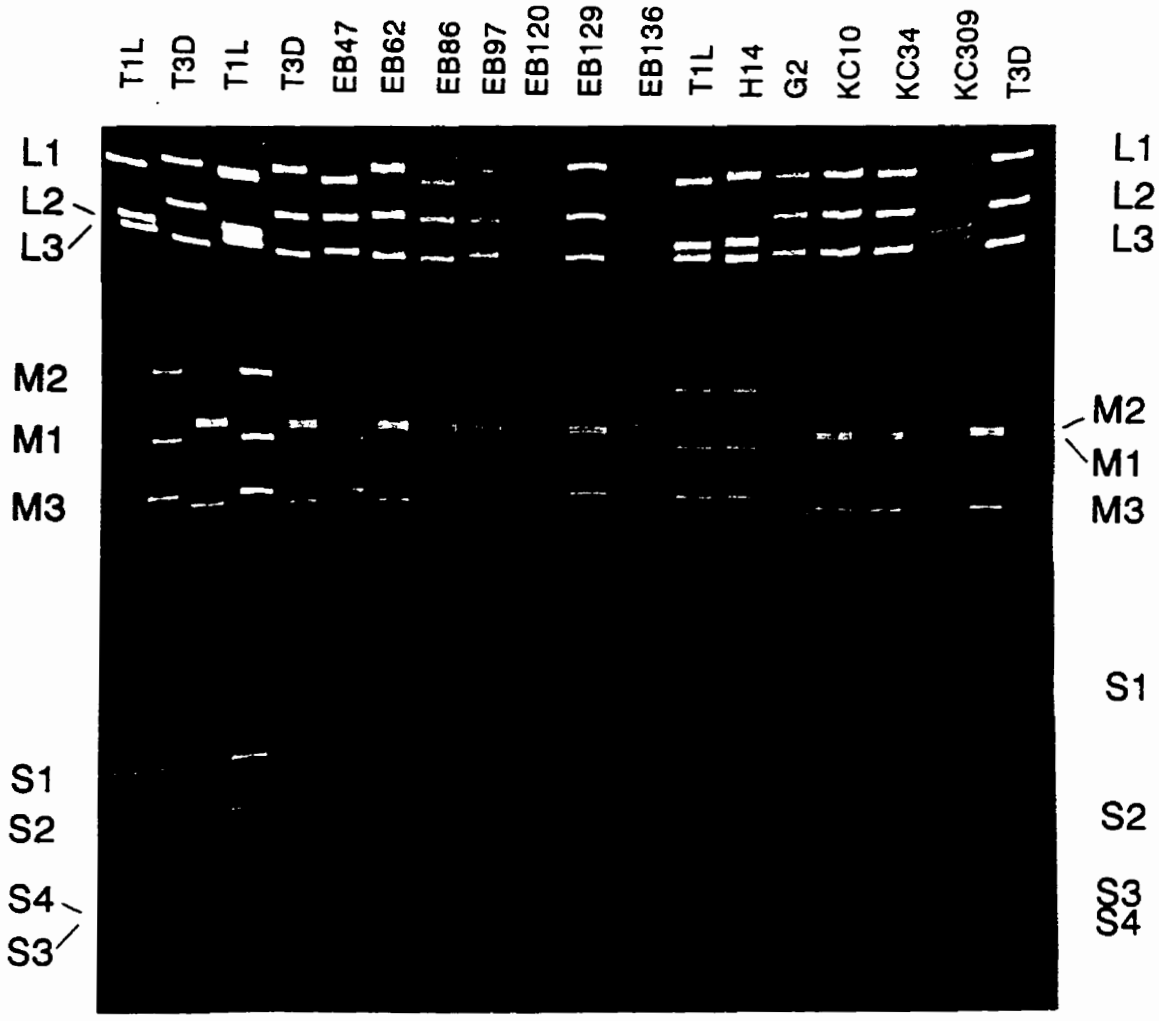

T1L
T3D
T1L
T3D
EB47
EB62
EB86
EB97
EB120
EB129
EB136
T1L
H14
G2
KC10
KC34
KC309
T3D
L1
L2
L3
M2
M1
M3
S1
S2
S4
S3
L1
L2
L3
M2
M1
M3
S1
S2
S3
S4

Fig. 4. Genotypes of T1L x T3D inter-typic reassortants. The dsRNA genomes of T1L, T3D and their reassortants extracted by phenol/chloroform method as described in Section 2.3 were resolved in 10% SDS-PAGE. The genotypes of these reassortants were deduced and listed in Table 2. For example, note that in reassortant EB47, the uppermost gene (L1) migrates the same as does the L1 gene in T1L, the next gene (L2) of EB47 has the same mobility in the gel as does the L2 gene of T3D. The other eight genes of EB47 all migrate the same as do those of T1L. Therefore, the genotype of this reassortant, EB47 is 1, 3, 1, 1, 1, 1, 1, 1, 1, 1 as shown in Table 2. Representative clones shown in this figure are EB47, EB62, EB86, EB97, EB120, EB129, EB136, H14, G2, KC10, KC34 and KC309.

Table 2. Genotypes of T1L x T3D inter-typic reassortants deduced from SDS-PAGE

	Gene Segment									
Clone	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4
T1L	1	1	1	1	1	1	1	1	1	1
T3D	3	3	3	3	3	3	3	3	3	3
EB47[a]	1[b]	3	1	1	1	1	1	1	1	1
EB62	3	3	3	3	3	3	3	1	3	1
EB86	1	3	3	3	3	1	3	3	3	1
EB97	3	3	1	3	3	3	3	3	3	1
EB120	3	3	3	1	1	3	3	3	1	1
EB129	3	3	3	3	3	1	3	1	1	3
EB136	3	3	3	1	3	1	3	3	3	3
H14	1	1	3	1	1	1	1	3	3	1
G2	1	3	1	1	1	1	3	1	1	1
KC10	1	1	1	1	1	1	1	3	1	1
KC34	1	3	1	3	3	3	1	3	3	3
KC309[c]	3	1	3	3	3	1	3	3	3	1

a: T1L x T3D inter-typic reassortants resulted from a co-infection of T1L and T3D.

b: Numbers indicate parental source of gene. 1: T1 Lang; 3: T3 Dearing.

c: Clone was derived from a T3D x H14 cross (Coombs,et 1990).

reoviruses were dialysed in 1 X dialysis buffer (0.15M NaCl, 0.01M MgCl and 0.01M Tris, pH 7.4). Then, virus concentrations were measured and adjusted accordingly. It has been shown that the concentrations of viruses are critical for the conversion of reovirus virion to core particles (Drayna and Fields, 1982). Previous investigators also found that different serotypes require different optimum virus concentrations for core production; $\geq 5 \times 10^{13}/ml$ for T1L and $\geq 5 \times 10^{12}/ml$ for T3D. In addition, the M2 gene (encoding outer capsid protein $\mu 1/\mu 1C$) is responsible for this phenotypic variation between T1L and T3D (Drayna and Fields, 1982). Therefore, each digestion reaction contains 6.5 x 10^{13}PFU/*ml* gradient purified T1L viruses or reassortants with the M2 gene of T1L, or 2.5x10^{13}PFU/*ml* T3D viruses or reassortants with the M2 gene of T3D and 200μg/*ml* chymotrypsin. The reactions were incubated at 37^{0}C for 3 hours, then loaded onto CsCl gradients as detailed in Section 2.5. In initial experiments, T1L virus digestions generated two bands close to each other in CsCl gradient. Both bands were near the expected core positions, with densities of 1.43 (T1L lower band) and 1.40g/*ml* (T1L higher band) respectively. One band of T3D cores with a density of 1.43g/*ml* was found in CsCl gradient. The bands were collected and examined by electron microscopy. The particles from T3D and T1L lower band were confirmed to fit standard criteria of reovirus core particle: evenly dispersed, spherical and about 60 nm in diameter with the $\lambda 2$ spikes

present at the axes of five fold symmetry (Fig. 5A and B). Electron microscopy showed that the purified particles from T1L upper band has similar morphological characteristics as those from T3D and T1L lower band, except that there was extra material surrounding these particles (Fig. 5C, arrow head). In order to better characterize these particles, the purified particles from T1L lower and higher bands, and the single T3D band, were analyzed by SDS-PAGE (Fig. 6). Only four core proteins ($\lambda 1$, $\sigma 2$, $\lambda 3$ and $\mu 2$) and pentameric spike protein $\lambda 2$ were found in the particles from T1L lower band and T3D (Fig. 6, lane 3, 5). Extra unidentified proteins with smaller molecular weights than $\sigma 2$ were present in the T1L higher band particles (Fig. 6, lane 4). These protein bands may represent digested fragments of outer shell proteins. To test this possibility, Western blot assays were performed as detailed in Section 2.6. These small proteins only reacted with anti-whole virus and anti-$\mu 1/\mu 1C$ antiserum but not anti-$\sigma 3$ antiserum, suggesting that these fragments are the digested products of major outer shell protein $\mu 1/\mu 1C$ (Fig. 7).

It has been suggested that the conversions of cores from virions have two phases: from virions to ISVPs (protease dependent); from ISVPs to cores (particle and ionic concentration dependent) (Joklik, 1972). Therefore, the incomplete digestion of $\mu 1/\mu 1C$ could be attributed to factors such as enzyme concentration, or ionic strength in the reactions. Various digestion conditions were tested and the

optimum conditions were determined. First, after CsCl gradient purification, viruses were dialysed against 1 X SSC(150*mM* NaCl, 15*mM* Sodium Citrate, pH 7), instead of dialysis buffer (0.15M NaCl, 0.01M MgCl and 0.01M Tris, pH 7.4). Secondly, the chymotrypsin concentration was decreased to 100*μg/ml* from the previous 200*μg/ml*. Finally, 0.1*mM* CsCl was added into reaction which may facilitate the core conversion (Joklik, 1972). Only one band each for T1L and T3D was observed in CsCl gradient after protease digestion done under above conditions. Electron microscopy showed that they were normal core particles, without any attached material (Fig. 5, D-E). In addition, no small digested fragments were seen in SDS-PAGE (Fig. 6, lane 6-7). Therefore, all subsequent experiments were performed under these modified conditions, including dialysis against 1 X SSC, 100*μg/ml* chymotrypsin and 0.1 mM CsCl.

3.3. Establishment of optimum conditions for reovirus *in vitro* transcriptase assay using gradient purified core particles. Previous investigators used purified whole virion to perform *in vitro* transcriptase assays. Purified virions were first "activated" by removing outer capsid proteins with proteases to generate core particles. Then, the various substrates were added into the reactions to synthesize ssRNAs (Skehel and Joklik, 1969; Drayna and Fields, 1982). The general reaction conditions that allow reovirus to generate

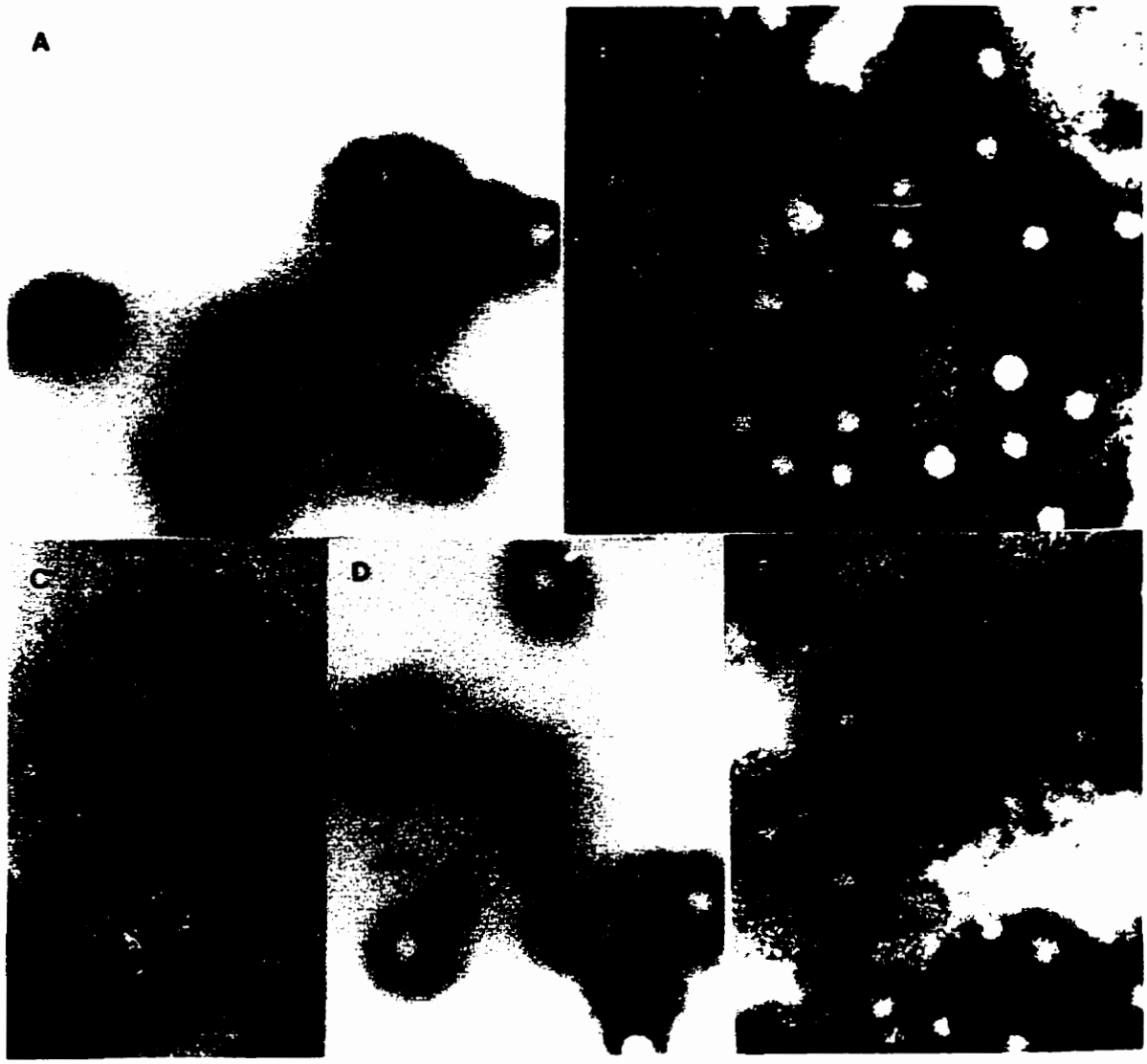

A
C
D

Fig. 5. Appearance of purified T1L and T3D particles digested under different conditions. Purified T1L ($6.5 \times 10^{13}/ml$) or T3D ($5 \times 10^{12}/ml$) viruses were either dialysed against 1 X dialysis buffer, then digested with 200$\mu g/ml$ chymotrypsin at 37^{0}C for 3 hours (A-C) or dialysed against 1 X SSC, then digested with 100 $\mu g/ml$ chymotrypsin and 0.1 mM CsCl at 37^{0}C for 3 hours (D-E). Digested products were then loaded onto CsCl gradients as detailed in Section 2.5. Particle bands were collected, negatively stained with Phosphotungstic Acid, and observed. Particles from T3D band (A), T1L lower band (B), T1L higher band (C), T3D (D) or T1L (E) core particles digested under modified conditions were as shown. All images shown are at a magnification of 100,000x. Arrow head in (C) shows extra material surrounding particles purified from T1L high band.

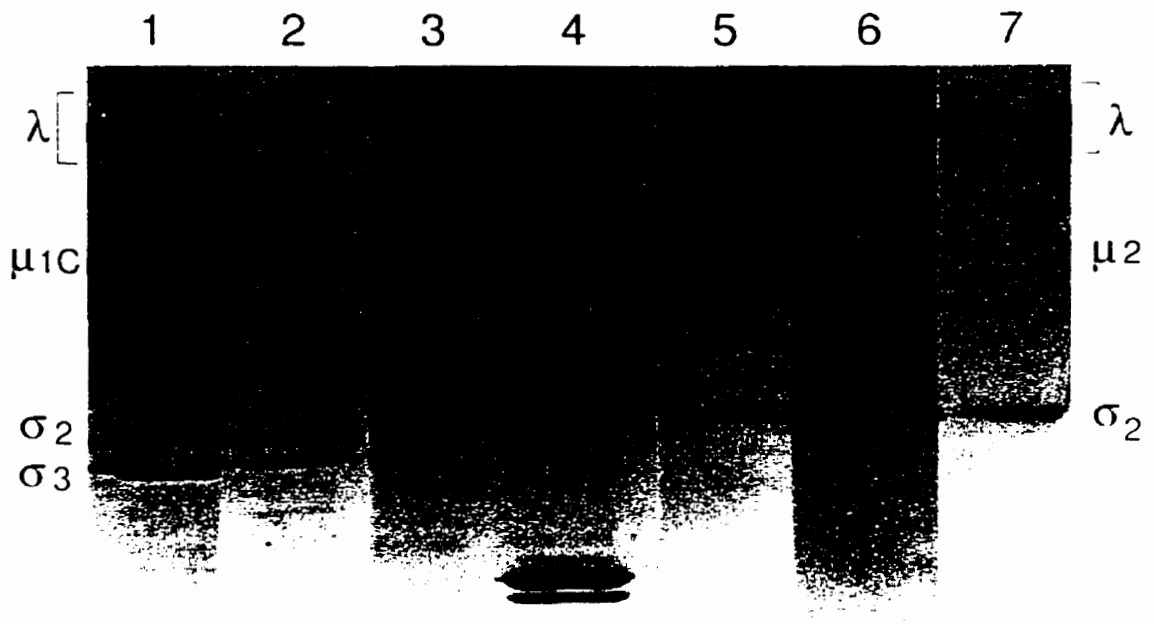

1
2
3
4
5
6
7
λ
μ1C
σ2
σ3
λ
μ2
σ2

Fig. 6. SDS-PAGE of purified particles digested under different conditions as described in the Fig. 5 legend. Samples were resolved by 5-15% SDS-PAGE (7 x 8 x 0.1 cm) at 18 mA for 1 hour and stained with Coomassie Brilliant Blue as detailed in Section 2.12. Purified T1L (lane 1) and T3D viruses (lane 2) were used as viral protein markers. T1L low core band (lane 3), T1L high core band (lane 4), T3D core band (lane 5), or T1L or T3D core particles digested under modified conditions, including dialysis against 1 X SSC, 100$\mu g/ml$ chymotrypsin and 0.1 *mM* CsCl (lane 6, 7).

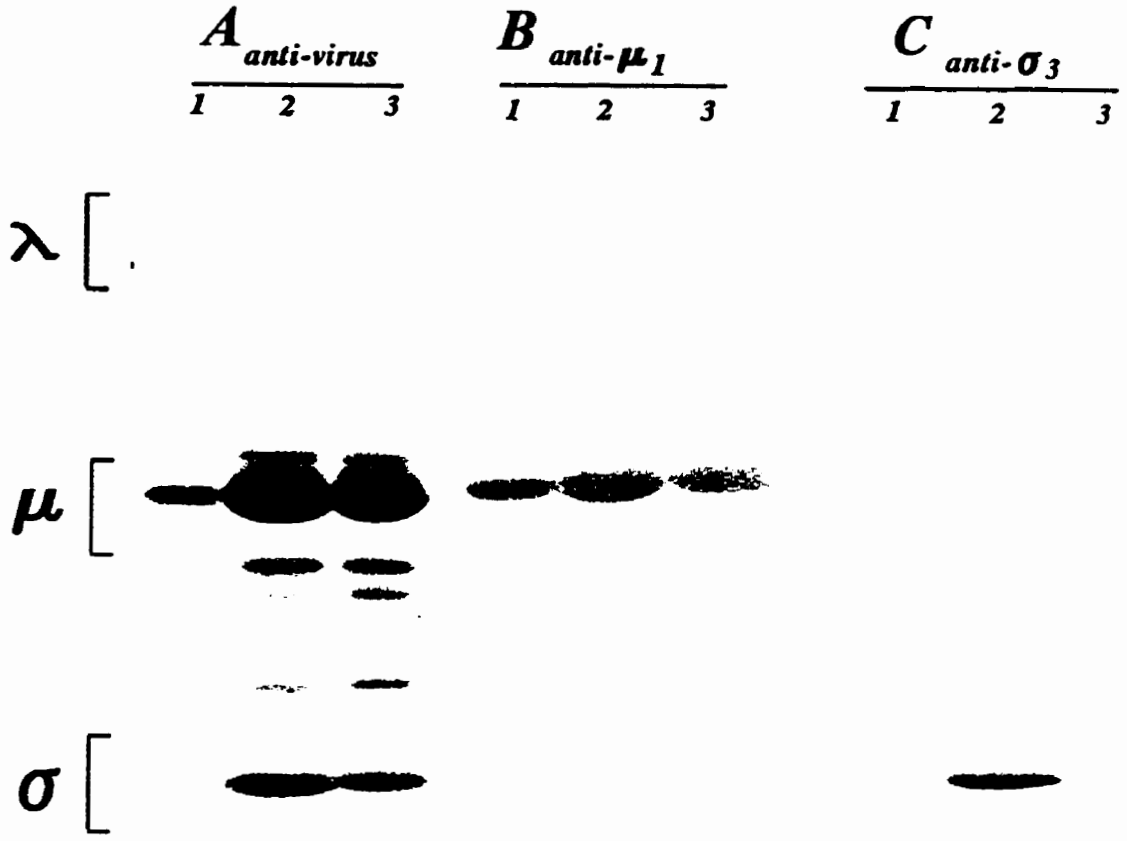

A anti-virus
1 2 3
B anti-μ1
1 2 3
C anti-σ3
1 2 3
λ
μ
σ

Fig. 7. Western Blot analyses of gradient purified particles from T1L high band. T1L high band particles obtained as described in the Fig. 5 legend (lane 1), and T3D virus (lane 2) or T1L virus (lane 3) were resolved in 5-15% SDS-PAGE (16.0 x 12.0 x 0.1) at 20 mA for 6 hours as detailed in Section 2.6. The gels were collected and soaked in TransBlot buffer for 20 minutes. Each transferring sandwich in the order of: (from bottom to top) one filter paper, membrane pre-soaked in TransBlot buffer, gel and another filter paper was placed onto a transfer cell. The transfer of proteins from SDS-PAGE to membranes was conducted at 15V for 30 minutes. After transfer, membranes were washed with dH_2O, then stained with 2% Ponceau S. Then, membranes were rinsed with dH_2O 3 times and soaked in Blocking Buffer for 2 hours. Various rabbit antisera, anti-whole virus antiserum (A), anti-μ1 antiserum (B) and anti-σ3 antiserum (C) were used to incubate different membranes for 90 minutes. Then, anti-rabbit IgG coupled with Horse Radish Peroxidase (HRP) were added into reactions and incubated for 90 minutes. The membranes were washed with TBST four times. The protein bands were developed by treating membranes with staining solution including 0.5% DAB. When the bands were of the desired intensity, membranes were washed with dH_2O, then dried between multiple layers of absorbent papers.

acid-insoluble nucleic acid have been described. These include removal of the outer viral capsid, a sharp Mg^{2+} optimum, pH optimum, and the presence of phosphoenol pyruvate, pyruvate kinase, and all four nucleoside triphosphates. Since core particle contains all the necessary enzymes required for transcription (Chang and Zweerink, 1971; Hay and Joklik, 1971; Shatkin, 1974; Furuichi *et al.*, 1975), *in vitro* transcriptase assays used in this study were performed using gradient purified core particles instead of whole virions. Therefore, it was necessary to determine the optimum conditions for this modified assay to compare them with previous studies.

3.3.1. Reovirus core transcription rate is concentration dependant. Intact virus particles did not produce significant amounts of transcription product (Fig. 8B, □), in agreement with the observations of others (Skehel and Joklik, 1969; Drayna and Fields, 1982). Prior transcriptase mapping experiments started with purified virions that were "activated" by serotypically-dependant different methods, one of which was particle concentration (Drayna and Fields, 1982). Thus, we first evaluated the effect of purified core particle concentration on transcriptase ability. The efficiency of core transcription was dependant upon concentration of particles (Fig. 8). At T1L core concentrations of ≤ 7.5 x 10^9 per μl and at a temperature of 42°C, the conversion of [^{32}P]-UTP into acid insoluble material proceeded at a rate of

nearly 50 CPM per 10^8 particles per hour and was linear over at least 3 hours. For example, the absolute amount of radioactive material synthesized by cores at a concentration of 8.5 x $10^8/\mu l$ was measured at 18,700 CPM (Fig 8, ■). At concentrations > 7.5 x 10^9 per μl cores produced less TCA-precipitable material per particle. For example, cores at a concentration of 2.3 x $10^{10}/\mu l$ only produced 12 CPM per 10^8 particles per hour (Fig. 8). Since radio-labelled material made by these cores linearly increased over 3 hours, this decrease in ratio of CPM/particle/hour did not result from limiting reaction precursors (data not shown). By contrast, core particles at a concentration of 7.5 x $10^9/\mu l$ synthesized as much as 150,000 CPM TCA-precipitated material and had the same CPM/particles ratio (50 CPM/10^8/h) as did the cores at lower concentration (Fig. 8, ▨). In addition, when the final concentration of ^{32}P-UTP was between $0.045\mu Ci/\mu l$ and $0.45\mu Ci/\mu l$, the amount of label incorporated into acid insoluble material was linearly proportional to the concentration of ^{32}P-UTP used (data not shown). Core particles derived from T3D gave similar results; at concentration < 7.5 x$10^9/\mu l$, cores produced high CPM per core particle ratios but low absolute amount of radio-labelled material; at concentrations > 7.5 x 10^9 per μl they produced significant [^{32}P]-UTP labelled material but low TCA-precipitable material per particle. Only at a concentration of 7.5 x $10^9/\mu l$, core particles synthesized radio-labelled material at both high

absolute amount and high CPM/particle ratio. Therefore, to maintain high sensitivity and reproducibility core concentrations of all clones examined was kept at about 7.5 x 10^9 per μl and ^{32}P-UTP was routinely used at 0.1$\mu Ci/\mu l$ in subsequent experiments.

3.3.2. The Mg^{2+} concentration of 9.5 *mM* is the optimum Mg^{2+} concentration for reovirus transcriptase. *In vitro* transcriptase assays were performed to determine optimum Mg^{2+} concentration for reovirus transcriptase. Both T1L and T3D displayed a Mg^{2+} optimum at 9.5 *mM* (Fig. 9), in agreement with previous studies (Joklik, 1972; Drayna and Fields, 1982). Therefore, a concentration of 9.5 *mM* Mg^{2+} was used in all subsequent experiments.

3.3.3. The optimum pH value for reovirus transcriptase is pH 8.0. Previous studies have shown that reovirus transcribes most efficiently between pH values of 8.0 and 9.0 (Skehel and Joklik, 1969; Drayna and Fields, 1982). In addition, Drayna and Fields showed that there were subtle strain-specific differences in the pH optimum within this range (Drayna and Fields, 1982). Thus, the transcriptase reactions with purified cores were conducted at multiple temperatures and at several pH values between 8.0 and 9.0. Purified T1L and T3D cores synthesized ssRNAs most efficiently at about 50^{0}C and there was a different temperature optimum between T1L and T3D

at pH 8.0. At high pH values, both T1L and T3D produced less ssRNAs and the strain-specific differences at temperature optimum were less obvious (Fig. 10). Therefore, a pH value of 8.0 was used in all following experiments.

3.4. T1L and T3D cores have different temperature optima for transcription. Based on prior optimization results (Section 3.3.1-3.3.3), the transcriptase assays were performed under following conditions including 7.5 x10^9/μl purified T1L or T3D core particles, 9.5 *mM* Mg^{2+} and pH 8.0. (The [^{32}P]-UTP labelled material synthesized by cores from these *in vitro* transcriptase assays will later be shown as the 10 full-length functional viral ssRNAs in Section 3.11, indicating that the *in vitro* transcriptase assays used in this study truly measured reovirus transcriptase activity.) The *in vitro* trascriptase assays showed the reovirus transcriptase has an unusual temperature profile (Fig. 10, 11). Purified T1L and T3D cores had little detectable transcriptase activity at temperatures of $\leq$ 30°C (Fig. 10, 11). Both serotypes showed transcriptase activity at higher temperatures, with the peak of activity at about 50°C, as previously reported (Skehel and Joklik, 1969; Drayna and Fields, 1982). However, T1L cores transcribed most efficiently at 48°C and T3D cores transcribed most efficiently at 52°C. The absolute amount of radioactivity incorporated into acid precipitable material varied greatly from experiment to experiment, contributing to

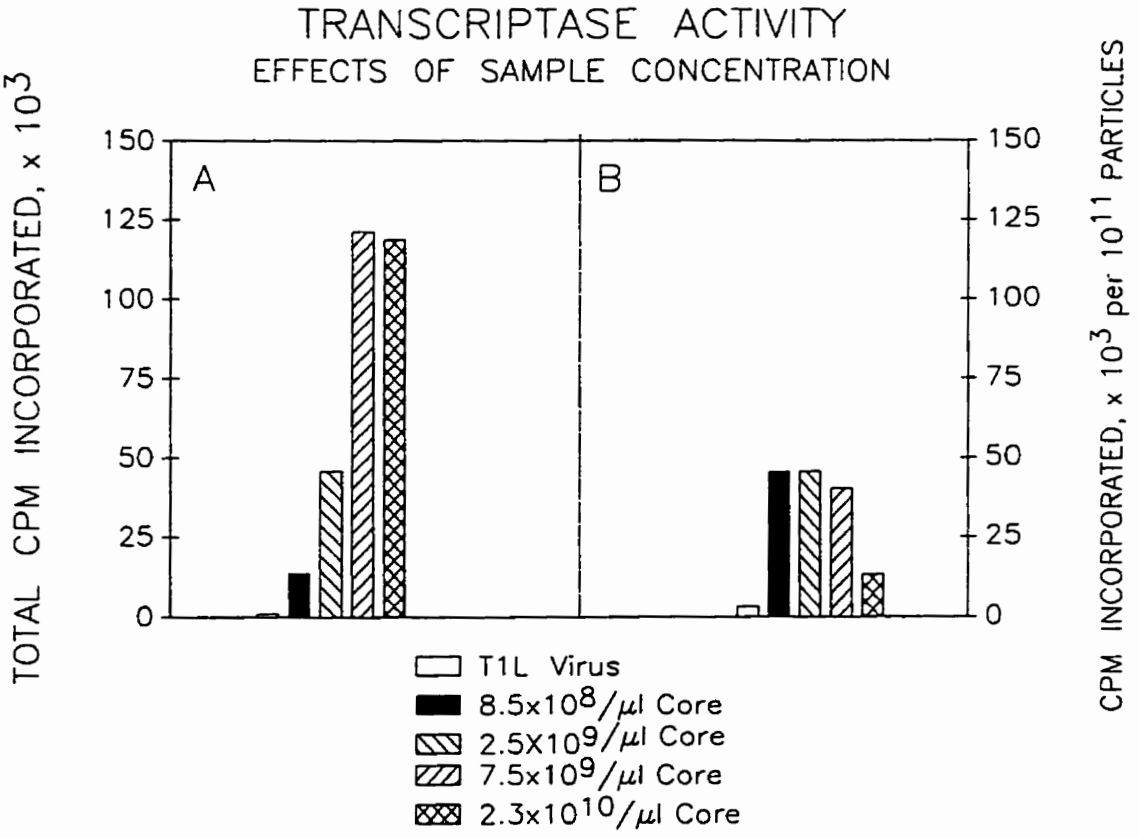

TRANSCRIPTASE ACTIVITY
EFFECTS OF SAMPLE CONCENTRATION
TOTAL CPM INCORPORATED, x 10^3
CPM INCORPORATED, x 10^3 per 10^11 PARTICLES
A
B
150
125
100
75
50
25
0
T1L Virus
8.5x10^8/μl Core
2.5X10^9/μl Core
7.5x10^9/μl Core
2.3x10^10/μl Core

Fig. 8. Effect of particle concentration on the transcriptase activity of purified reovirus T1L cores. Transcription reactions were set up as detailed in Section 2.7. Aliquots of T1L cores at the indicated final concentrations (in a reaction volume of 44μl) were incubated with NTP's and 0.045μCi per μl of α-^{32}P UTP at 42°C for 1 hour. Samples were precipitated with TCA, precipitates collected on filters, and counted. □, T1L virus at 7.5 x 10^9 per μl; ■, T1L cores at 8.35 x $10^8/\mu l$; ▨, T1L cores at 2.5 x $10^9/\mu l$; ▨, T1L cores at 7.5 x $10^9/\mu l$; ▩, T1L cores at 2.25 x $10^{10}/\mu l$. A) Total incorporation into acid-insoluble counts; B) ^{32}P UTP incorporation (from panel "A") expressed as quantity incorporated per 10^{11} particles.

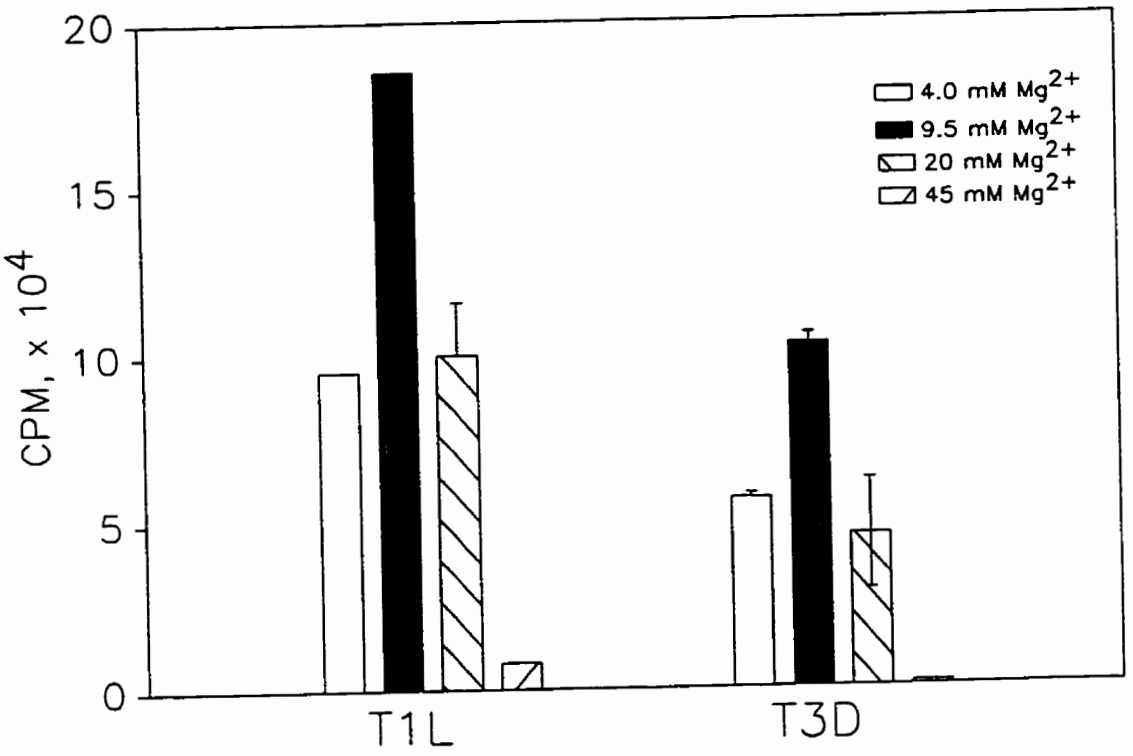

20
15
10
5
0
CPM, x 10^4
4.0 mM Mg^{2+}
9.5 mM Mg^{2+}
20 mM Mg^{2+}
45 mM Mg^{2+}
T1L
T3D

Fig. 9. Effect of Mg^{2+} concentration on the transcriptase activity of purified reovirus T1L and T3D cores. Transcription reactions were set up as detailed in Section 2.7. Aliquots of T1L or T3D cores (7.5 x $10^9/\mu l$) at indicated concentrations of $MgCl_2$ were incubated with NTP's and 0.1 μCi per μl of α-^{32}P UTP at 48°C for 1 hour. Samples were precipitated with TCA, precipitates collected on filters, and counted. ⊡ Mg^{2+} at 4.0mM; ■ Mg^{2+} at 9.5mM; ▨ Mg^{2+} at 20mM; ▩ Mg^{2+} at 45mM;

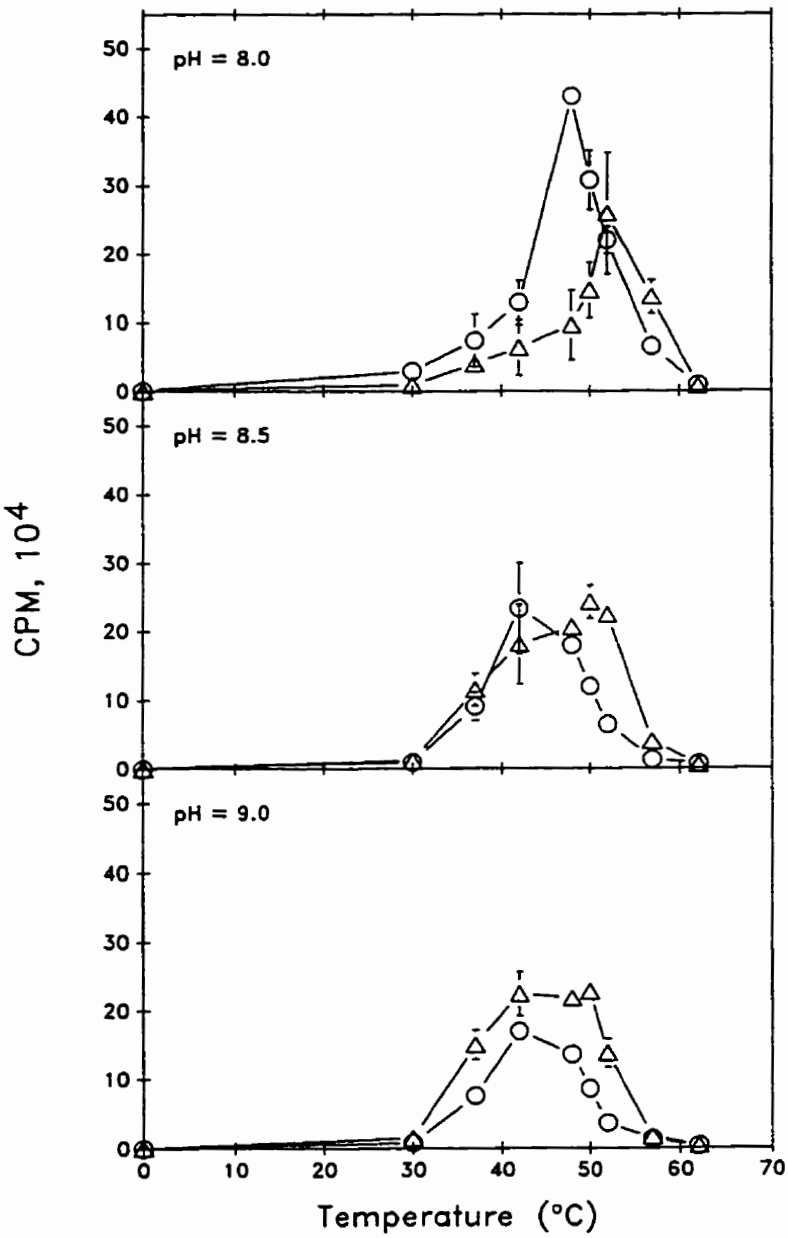

pH = 8.0
pH = 8.5
pH = 9.0
CPM, 10^4
Temperature (°C)
0
10
20
30
40
50
60
70

Fig. 10. Transcriptase activities of purified reovirus cores at different temperatures and pH values. Aliquots of 3.5 x 10^{11} total core particles in 50μl volumes were incubated with 0.1μCi of [α^{32}P]UTP per μl at the indicated temperatures and pH values for 1 hour and precipitated with 5% TCA. The precipitates were collected on filters and counted. Results are the average of duplicate experiments. ○, T1L; △, T3D. Error bars indicate variability (standard deviations) between experiments.

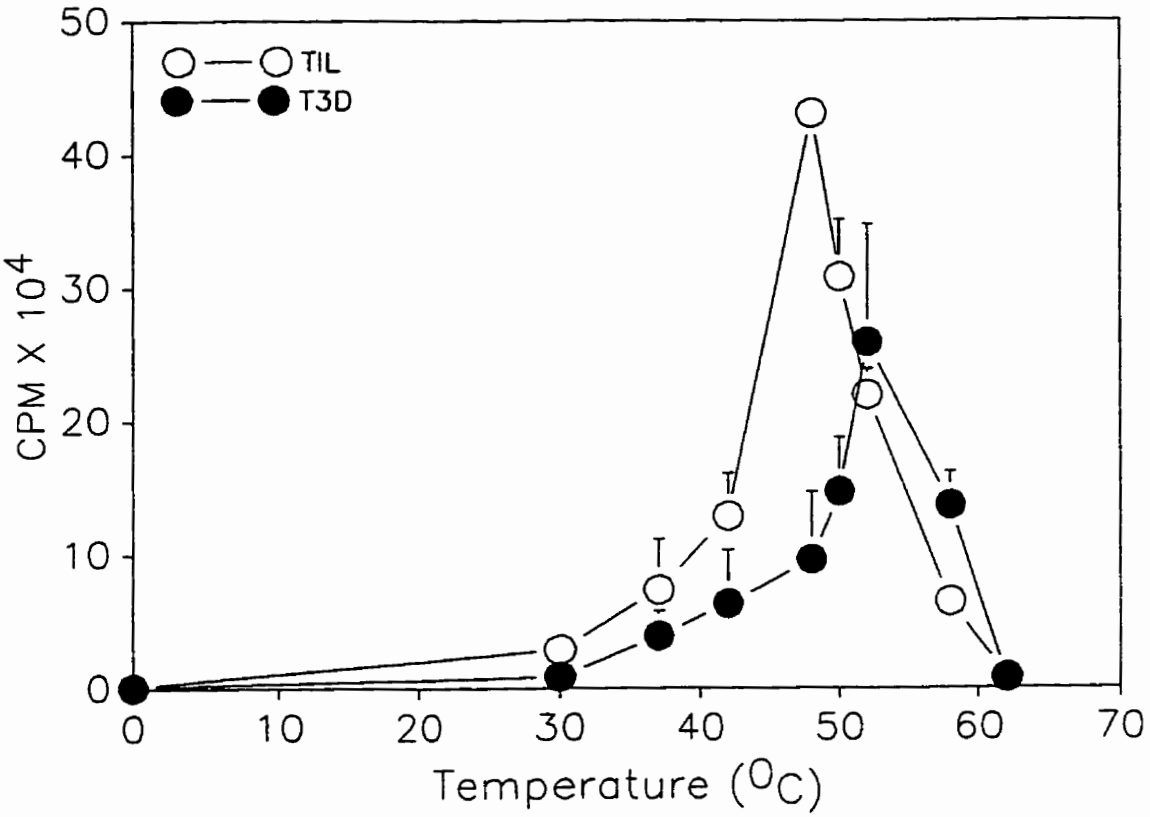

TIL
T3D
CPM X 10^4
Temperature (^{0}C)
0
10
20
30
40
50
60
70

Fig. 11. Transcriptase activity of purified reovirus cores at different temperatures. Reactions were set up as detailed in the legend to Fig. 8, but using α-^{32}P UTP at 0.1μCi per μl. Aliquots of 3.5 x 10^{11} total core particles in 50μl volumes were incubated at the indicated temperatures for 1 hour, precipitated with TCA, precipitates collected on filters, and counted. Results are the average of duplicate experiments. o, T1L; •, T3D. Error bars indicate variability (standard deviations) between experiments.

the relatively large error bars. However, the pattern remained the same in any given experiment; the T1L peak activity was < 50°C and the T3D peak activity was > 50°C. In addition, T1L cores consistently generated more acid-precipitable material at and near its activity peak than did T3D cores. Neither type of core produced significant amounts of product at temperatures ≥ 62°C. This observation will be followed up on in later section 3.9-3.14 of this thesis.

3.5. The M1 gene is associated with differences in the temperature optimum of transcription. The results in Section 3.4 demonstrated that T1L and T3D had different temperature optima for transcriptase activity (Fig. 10, Fig. 11). Therefore, T1L x T3D inter-typic reassortants could be used to determine which gene(s) was/were responsible for this phenotypic differences. Core particles of T1L X T3D reassortants were purified and used in *in vitro* transcriptase assays as described above. Some reassortants (*ie.*, G2) behaved like T1L; cores derived from such clones possessed more activity at 48°C than they did at 50°C and more activity at 50°C than at 52°C (Fig. 12). Other reassortants (*ie.*, KC34) behaved like T3D; they had more activity at 52°C than at 50°C and more activity at 50°C than at 48°C. A few reassortants (*ie.*, EB86 and EB129) behaved like neither parent; they had virtually the same activity at 48°C as at 52°C, with a minor peak of activity at 50°C. The different

reassortant behaviors prevented all clones from easily being placed into only two distinct groups (because some of the reassortants had intermediate behavior) (see Section 1.2). Therefore, the relative transcriptase activities at various temperatures were determined in order to facilitate reassortant mapping analyses. This relative activity could be represented as a ratio of the transcriptase activity at 48°C divided by the transcriptase activity of the same clone at 52°C. The average ratio values obtained for each reassortant clone from three or more experiments generated a continuum from about 1.8 to about 0.5 (Table 3). Many of the reassortants near the top of the table (*ie.*, G2, H17, and EB120) behaved like T1L; the 48°/52°C ratios from every experiment were > 1.0. The relatively large errors associated with G2 and H17 resulted from single experiments in which the activity ratios were significantly higher. Many reassortants near the bottom of the table (*ie.*, EB62, G16, and KC34) had the same characteristics as that of T3D; the 48°/52°C ratios were always less than 1.0. A few reassortants (*ie.*, EB47, EB97, and KC309) gave variable results with 48°/52° ratios less than 1.0 in some experiments and greater than 1.0 in other experiments. However, the average ratio obtained for these reassortants placed them within the continuum. The reassortants were ranked according to the average activity ratio values obtained. A non-parametric Wilcoxon rank sum analysis of the relative contribution of each of the genes of

all ranked reassortants shown in Table 3 indicated that the M1 gene was strongly associated (P = 0.0003) and that no other genes were associated with the difference in temperature optimum (Table 4). A parametric linear regression analysis of each of the 10 genes indicated that the M1 gene accounted for 74% of the variance (R^2 = 0.74), whereas the S3 gene contributed to 9% of the variance (R^2 = 0.09), the S4 gene accounted for 5% of the variance (R^2 = 0.05), and each of the other genes accounted for less than 4% of variance. All subset multiple regression analysis showed that 90% of the variance (R^2 = 0.9) could be attributed to the 10 gene segments and that 74% of the total variance (R^2 = 0.74) could be attributed solely to the viral M1 gene. The viral S1 gene added a contribution of 6% to the explained variance (R^2 = 0.06) whereas each of the other genes added less than 4% to the explained variance. A Wilcoxon analysis of the entire data set of individual experimental results increased the significance of the M1 gene (P = 0.0001) and suggested moderate significance of the viral M2, S3, and S4 genes (P = 0.02, P = 0.003, and P = 0.008, respectively). Linear regression analysis of each of the 10 genes in the set of individual experimental value indicated that the M1 gene accounted for 37% of the variance (R^2 = 0.37), the L3 gene accounted for 5% of variance, the M2 gene accounted for 6% of variance, the S3 gene accounted for 10% of variance, the S4 gene accounted for 7% of variance, and each of the other genes

accounted for less than 4% of variance. All subset multiple regression analysis showed that 44% of the variance (R^2 = 0.44) could be attributed to the 10 gene segments and that 37% of the total variance (R^2 = 0.37) could be attributed solely to the viral M1 gene. No other viral gene added an adjusted contribution greater than 4% to the explained variance (Table 4).

3.6. The L1 and M1 genes are associated with differences in transcriptase efficiency. The prior results in Section 3.4 revealed that T1L consistantly had higher cumulative transcriptase activity near the activity peak (between 48°C and 52°C) than the cumulative activity of T3D cores (Fig. 10, Fig. 11). To identify the viral gene(s) responsible for this difference in transcriptase efficiency, I examined the cumulative activity of various reassortants in this temperature range. Preliminary analyses that involved summing and ranking the activities of each clone in Table 3 at each tested temperature failed to implicate any single gene as a determinant of transcriptase efficiency, but did suggest multiple genes might be involved (data not shown). Therefore, more reassortant core clones were prepared. In addition, to determine the cumulative transcriptase activity of each clone at multiple temperatures between 42°C and 57°C, reactions were conducted in an MJ Research model PTC-100 programmable thermal controller that had been

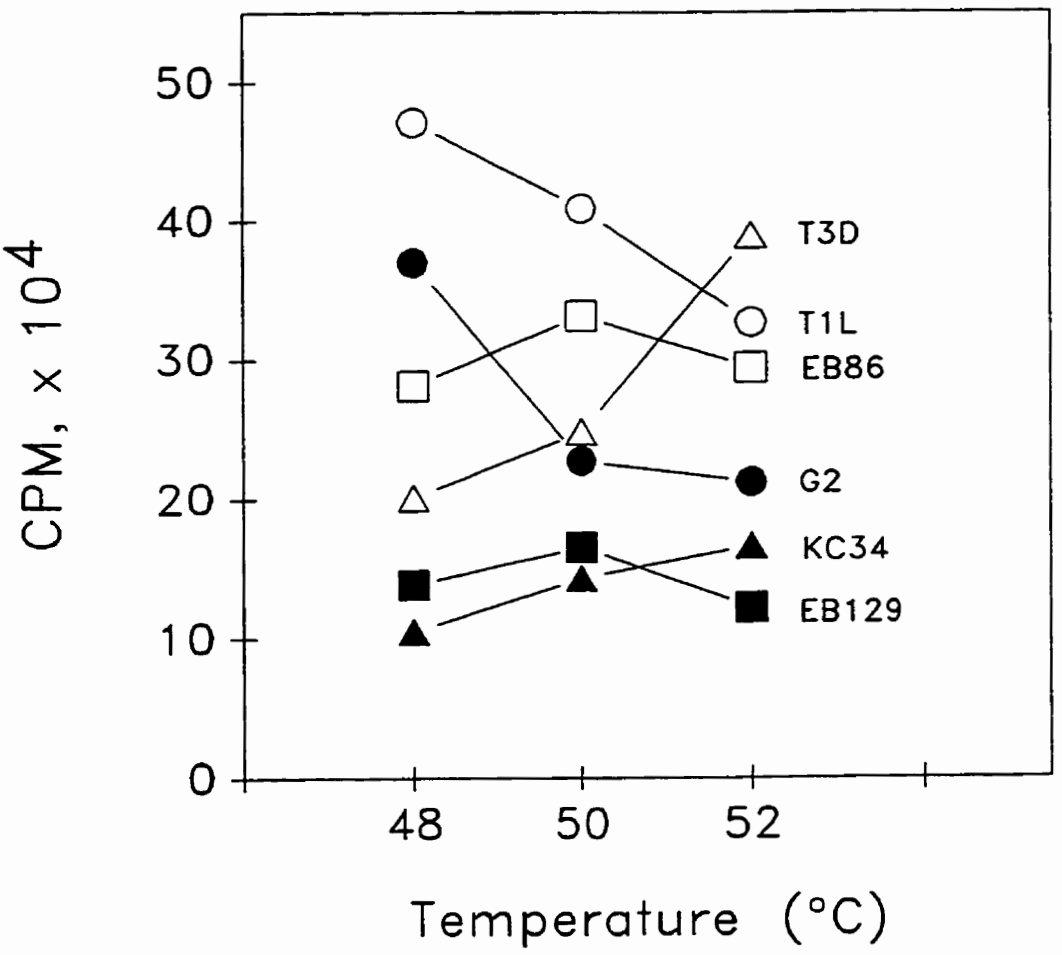

CPM, x 10^4
50
40
30
20
10
0
48
50
52
Temperature (°C)
T3D
T1L
EB86
G2
KC34
EB129

Fig. 12. Transcriptase activity of T1L, T3D, and selected T1L x T3D inter-typic reassortant cores at 48°C, 50°C, and 52°C. Purified cores from the indicated clones were assayed for transcriptase activity at the indicated temperatures as described in the legend to Fig. 11. Results are the average of three experiments (except T1L and T3D which are from five experiments).

Table 3. Genotypes of T1L x T3D inter-typic reassortants tested for transcriptase temperature optimum

	Electropherotype											
	Core					Outer Capsid			Non-Struc			
Gene:	L1	L2	L3	M1	S2	M2	S1	S4	M3	S3		
Clone Protein:	λ3	λ2	λ1	μ2	σ2	μ1	σ1	σ3	μNS	σNS	Ratio[a]	Rank[b]
G2	1[c]	3	1	1	1	1	3	1	1	1	1.773±1.149	19
H17	3	3	3	1	3	3	1	1	3	3	1.632±0.657	18
EB120	3	3	3	1	3	1	3	1	3	1	1.566±0.467	17
TIL	1	1	1	1	1	1	1	1	1	1	1.538±0.352	16
EB39	1	3	3	1	3	3	3	3	3	3	1.463±0.604	15
EB136	3	3	3	1	3	3	3	3	1	3	1.329±0.298	14
H14	1	1	3	1	3	1	1	1	1	3	1.288±0.175	13
EB47	1	3	1	1	1	1	1	1	1	1	1.261±0.551	12
KC10	1	1	1	1	3	1	1	1	1	1	1.244±0.345	11
EB129	3	3	3	3	1	3	3	3	1	1	1.207±0.256	10
EB97	3	3	1	3	3	3	3	1	3	3	1.015±0.169	9
EB86	1	3	3	3	3	3	3	1	1	3	0.989±0.054	8
EB62	3	3	3	3	1	3	3	1	3	3	0.902±0.087	7
EB146	1	1	1	3	1	1	1	3	1	1	0.682±0.117	6
KC309[d]	3	1	3	3	3	3	3	1	1	3	0.645±0.319	5
G16	1	1	1	3	3	1	1	1	1	1	0.632±0.145	4
KC34	1	3	3	3	3	3	1	3	3	3	0.611±0.215	3
EB96	1	3	1	3	1	1	1	1	1	3	0.569±0.149	2
T3D	3	3	3	3	3	3	3	3	3	3	0.538±0.218	1

a: Ratio derived by dividing 48°C activity by 52°C activity (Average, ± standard deviation of 3 or more experiments).
b: Ranking used for Wilcoxon rank sum analysis (Table 4).
c: Numbers indicate parental source of gene. 1: T1Lang; 3: T3Dearing.
d: Clone was derived from a T3D x H14 cross.

Table 4. Nonparametric and parametric analyses of the 10 reovirus gene segments involved in transcriptase temperature optimum

	Mean transcriptase optimum[a]		
	Univariate[b]		Multivariate[c]
Gene	Wilcoxon (P Value)	Linear Regression (R^2)	(R^2)
10 Genes			0.90
L1	0.97	0.0001	
L2	0.69	0.03	
L3	0.6	0.01	
M1	**0.0003**	**0.74**	0.74
M2	0.44	0.03	
M3	0.1	0.0002	
S1	0.71	0.01	0.06
S2	0.9	0.004	
S3	0.23	0.09	
S4	0.36	0.05	

a: Determined from gene rankings of averaged clones shown in Table 3.

b: Univariate tests of each individual gene were the nonparametric Wilcoxon rank sum analysis (expressed as P value) and the parametric linear regression analysis (expressed as R^2).

c: Multivariate test used was the all subset multiple regression analysis. Numbers shown represent the R^2 values of genes adjusted for all other genes in the selected model. A blank cell means $R^2 < 0.04$, not statistically significant, and not included in the model.

programmed to increase temperature from 42°C to 57°C over a two hour incubation (0.1°C increase every 48 seconds). T3D cores generated about 60% the amount of product as did T1L cores (Table 5). Reassortants generated varying amounts of product, from about twice the amount produced by T1L (clones H15 and H41) to about 9.5% the amount produced by T1L (KC309). A Wilcoxon rank sum analysis of the relative contribution of each of the genes of all ranked reassortants shown in Table 5 indicated that the L1 gene was significantly associated ($P = 0.01$), that the M1 gene was strongly associated ($P = 0.0002$), and that no other genes were associated with the difference in transcriptase efficiency (Table 6, left portion). Linear regression analysis of each of the 10 genes indicated that the M1 gene accounted for 33% of the variance ($R^2 = 0.33$), the L1 gene contributed for 13% of the variance ($R^2 = 0.13$), whereas the S1 gene accounted for 6% of the variance ($R^2 = 0.06$), the S3 gene added a contribution of 6% to the explained variance, and each of the other genes accounted for less than 4% of variance. All subset multiple regression analysis of the average transcriptase efficiencies showed that 51% of the variance ($R^2 = 0.51$) could be attributed to the 10 gene segments and that 33% of the total variance ($R^2 = 0.33$) could be attributed solely to the viral M1 gene. The viral L1 gene added a contribution of 6% to the explained variance and the S3 gene added an additional contribution of 5% to the variance, whereas each of the other genes added less than 4%

to the explained variance. A Wilcoxon analysis of the entire data set of individual experimental results increased the significance of both the L1 gene ($P = 0.0004$) and the M1 gene ($P = 0.0001$) and suggested moderate significance of the viral M3 and S1 genes ($P = 0.025$ and $P = 0.014$, respectively) (data not shown). Linear regression analysis of each of the 10 genes indicated that the M1 gene accounted for 27% of the variance ($R^2 = 0.27$), the L1 gene accounted for 10% of the variance ($R^2 = 0.10$), and each of the other genes accounted for less than 5% of variance. All subset multiple regression analysis of the individual transcriptase efficiencies showed that 40% of the variance ($R^2 = 0.40$) could be attributed to the 10 gene segments and that 27% of the total variance ($R^2 = 0.27$) could be attributed solely to the viral M1 gene. The L1 gene added a contribution of 5% to the explained variance and each of the other genes added less than 5% to the explained variance.

3.7. The ssRNA molecules produced by cores *in vitro* are stable at high temperatures. Both T1L and T3D cores completely lost their transcriptase activities at temperatures above 60^{0}C; no ssRNA molecules were detected by cores at such temperature (Fig. 10, Fig. 11). One possible explanation is that the ssRNAs made by cores are not stable at such high temperatures. To test this possibility, the ssRNAs synthesized from cores at 48^{0}C were incubated at high

temperatures ranging from 62 to 80°C for 1 hour. The amount of ssRNAs maintained at 48°C was considered as 100% (Fig. 13, □). More than 60% of the ssRNAs were stable and measured even after being heated at 80°C for 1 hour (Fig. 13, ⊠). This result suggests that ssRNA molecules produced by cores *in vitro* are stable at high temperatures as high as 80°C.

3.8. Heated reovirus cores are morphologically altered. Another possible reason why cores lost their transcriptase activities at high temperatures is that core structures were disrupted and enzymatic functions of cores were destroyed at such high temperatures. To test this hypothesis, we examined cores exposed at high temperatures by electron microscopy. T1L cores incubated at 48°C (the optimum temperature of transcriptase activity) (see Fig. 10, 11) had the same morphological characteristics as did the cores incubated at 0°C (Fig. 14). They are spherical and about 60 nm in diameter. The λ2 spikes are still present at their axes of five fold symmetry. In contrast to cores maintained at 0°C which are evenly dispersed and display no extruded material, cores incubated at 48°C with appropriate precursors generate extruded material that represents ssRNA (Fig. 14B arrow), an observation consistent with previous reports (Gillies *et al.*, 1971; Bartlett, N. M. *et al.*, 1974; Yeager *et al.*, 1997). The ssRNA material is not seen in the transcriptionally inactive core samples incubated at 0, 62, 65, or 70°C (Fig. 14A, C-E).

Table 5. The T1L x T3D inter-typic reassortants used to measure transcriptase activity

		Electropherotype											
			Core				Outer Shell			Non-Struc			
	Gene:	L1	L2	L3	M1	S2	M2	S1	S4	M3	S3		
Clone	Protein:	λ3	λ2	λ1	μ2	σ2	μ1	σ1	σ3	μNS	σNS	Scale[a]	Rank
KC26		3	3	1	1	1	1	3	3	3	3	222.9 ± 29.4	40
H15		1	3	3	1	3	3	3	1	3	3	204.3 ± 20.5	39
H41		3	3	1	1	3	1	1	1	3	3	175.3 ± 37.9	38
EB144		1	1	1	1	1	3	1	1	3	3	155.7 ± 6.7	37
EB39		1	3	3	1	3	3	3	3	3	3	138.7 ± 4.4	36
EB85		1	1	1	1	3	1	1	1	3	1	137.3 ± 5.9	35
EB96		1	3	1	3	1	1	1	1	1	3	131.3 ± 46.9	34
H14		1	1	3	1	3	1	1	1	1	3	126.0 ± 35.3	33
KC34		1	3	1	3	3	3	1	3	3	3	110.5 ± 43.4	32
KC36		3	1	1	1	3	1	1	3	1	1	107.5 ± 0.8	31
KC19		1	1	1	1	1	3	3	1	1	3	107.1 ± 42.9	30
EB93		1	1	1	1	1	1	1	1	1	3	103.0 ± 8.4	29
TIL		1	1	1	1	1	1	1	1	1	1	100.0 ± 0.0	28
G2		1	3	1	1	1	1	3	1	1	1	96.1 ± 7.7	27
EB47		1	3	1	1	1	1	1	1	1	1	93.9 ± 28.4	26
E3		3	3	3	3	3	3	1	3	3	3	89.8 ± 21.5	25
KC15		1	1	1	1	3	1	1	1	3	3	88.9 ± 3.4	24
EB73		3	3	3	3	3	3	3	1	1	1	86.7 ± 23.1	23
KC79		3	1	1	3	3	1	1	1	1	3	82.8 ± 19.6	22
H17		3	3	1	1	3	3	1	1	3	3	80.9 ± 6.5	21
KC298		1	1	1	3	3	1	1	3	1	3	78.6 ± 33.6	20
KC257		1	1	1	3	1	1	1	3	1	1	75.1 ± 24.4	19
EB138		3	1	1	3	3	3	3	1	1	1	74.7 ± 10.5	18
KC59		1	1	1	3	3	3	1	3	3	3	72.2 ± 18.2	17
EB86		1	3	3	3	3	3	3	1	1	3	70.9 ± 12.9	16
EB143		3	1	1	1	1	1	3	1	1	1	69.7 ± 32.5	15
KC252		3	1	3	3	1	3	1	3	1	3	64.9 ± 19.5	14
T3D		3	3	3	3	3	3	3	3	3	3	62.8 ± 11.3	13
KC3		1	3	1	3	1	1	1	1	3	1	62.0 ± 2.6	12
KC9		3	3	1	3	3	3	1	3	3	3	58.6 ± 1.1	11
EB62		3	3	3	3	1	3	3	1	3	3	58.1 ± 5.6	10
G16		1	1	1	3	3	1	1	1	1	1	57.8 ± 9.7	9
EB146		1	1	1	3	1	1	1	3	1	1	56.4 ± 6.6	8
KC10		1	1	1	1	3	1	1	1	1	1	50.8 ± 4.1	7
EB97		3	3	1	3	3	3	3	1	3	3	45.4 ± 4.9	6
H30		3	3	1	3	3	3	3	3	3	1	45.2 ± 4.9	5
EB129		3	3	3	3	1	3	3	3	1	1	44.8 ± 5.5	4
EB120		3	3	3	1	3	1	3	1	3	1	15.8± 1.6	3
KC309		3	1	3	3	3	3	3	1	1	3	9.5 ± 6.4	2
EB136		3	3	3	1	3	3	3	3	1	3	4.2 ± 4.6	1

a: Scale values were obtained by summing total incorporation from 42°C to 57°C in 2 hours (0.1°C/minute) for each clone in each of multiple experiments and dividing by the T1L incorporation in the same experiment (Average, expressed as percentage of T1L activity, ± standard deviation for 3 or more experiments)

Table 6. Nonparametric and parametric analyses of the 10 reovirus gene segments involved in transcriptase efficiency

	Mean transcriptase optimum[a]		
	Univariate[b]		Multivariate[b]
Gene	Wilcoxon (P Value)	Linear Regression (R^2)	(R^2)
10 Genes			0.51
L1	**0.01**	**0.13**	0.06
L2	0.72	0.01	
L3	0.77	0.0005	
M1	**0.0002**	**0.33**	0.33
M2	0.44	0.01	
M3	0.15	0.03	
S1	0.12	0.06	
S2	0.52	0.001	
S3	0.44	0.05	0.05
S4	0.34	0.03	

a: Determined from gene rankings of averaged clones shown in Table 3.

b: Univariate and multivariate tests used as described in Table2, footnotes c and d.

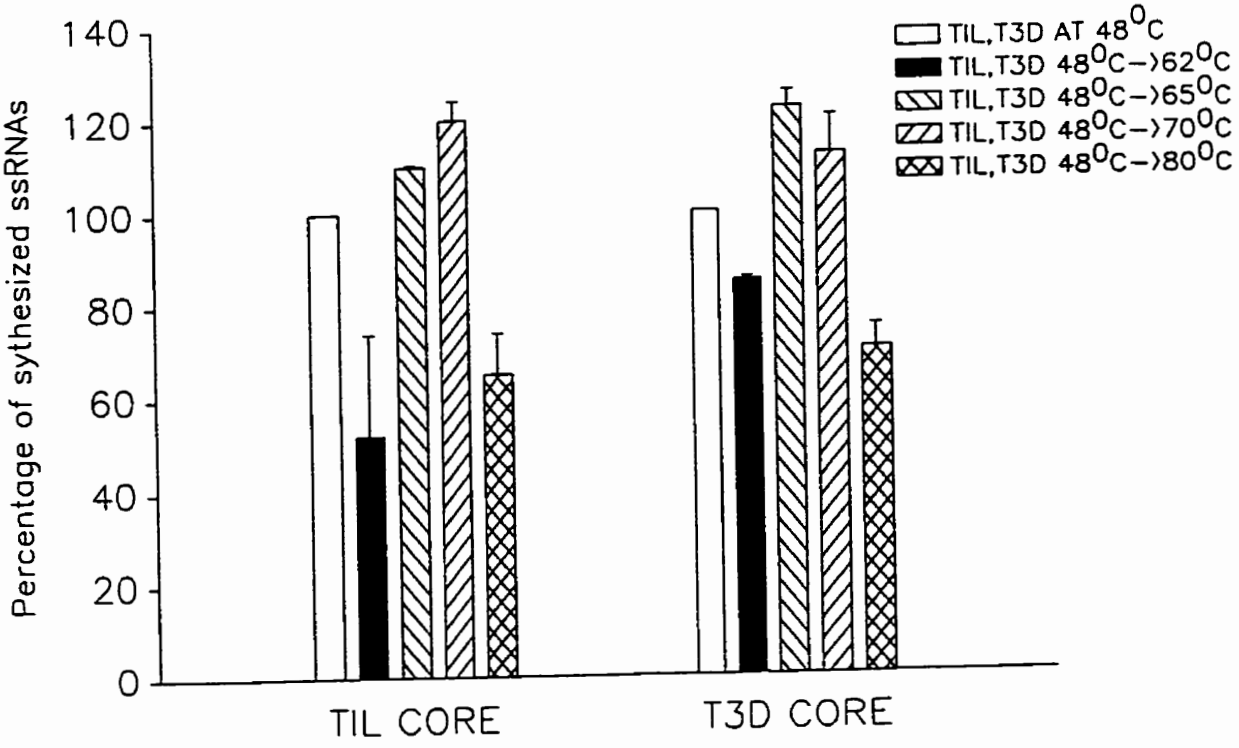

Percentage of sythesized ssRNAs
140
120
100
80
60
40
20
0
TIL CORE
T3D CORE
TIL,T3D AT 48°C
TIL,T3D 48°C->62°C
TIL,T3D 48°C->65°C
TIL,T3D 48°C->70°C
TIL,T3D 48°C->80°C

Fig. 13. Stability of synthesized ssRNAs at various high temperatures. Aliquots of 3.5 x 10^{11} total T1L or T3D core particles in 50μl volumes were incubated with 0.1μCi of [α^{32}P]UTP per μl at 48^{0}C for 1 hour. Then, the samples were re-incubated at various high temperatures for 1 hour. The reactions were terminated with 10% TCA for 30 minutes in ice. Transcripts were precipitated onto filters and counted. Radio-labelled material produced by the cores at 48^{0}C then exposed to high temperatures were compared to the ssRNAs made by the cores maintained at 48^{0}C (100%). Results are the averages of three individual experiments. Error bars indicate variability (SD) between experiments.□ ssRNAs incubated at 48^{0}C; ■ ssRNAs treated at 62^{0}C; ▧ ssRNAs heated at 65^{0}C; ▨ ssRNAs heated at 70^{0}C; ▩ ssRNAs treated at 80^{0}C;

Incubation of cores at 62°C had little effect on the structure of the capsid shell. A small proportion of the cores (~20%) were penetrated by the stain which suggests that they had lost their genomes (Fig. 14C arrow). In contrast, dramatic changes in capsid morphology occurred among those core particles heated at temperatures of 65°C and higher. Almost all of them lost their pentameric spikes and cavities can be clearly seen at the expected positions of the spikes (Fig. 14D arrow). Nearly 50% of the core particles incubated at 65°C still had genomes (Fig. 14D). However, virtually all cores heated at 70°C lost their spikes and genomes (Fig. 14E). In addition, large clumps were observed in the core samples heated at temperatures above 62°C. Similar observations were seen from heated T3D core particles (Data not shown).

3.9. Cores briefly exposed to 62°C are transcriptionally active, but the enzymatic activities of cores heated at 70°C are irreversibly lost. The morphological alterations associated with treatment of T1L or T3D core particles at elevated temperatures (Fig. 14) probably make the cores behave differently in aspects of their enzymatic activities, such as transcriptase activity. To test this possibility, cores were briefly exposed to various high temperatures, and then re-incubated at 48°C, the temperature optimum of transcription (see Section 3.4), for 1 hr to study

reversibility of the heat treatment on their transcriptase activities. The results are illustrated and summarized in Figures 15 and 16. In agreement with the data in Section 3.4 (Fig. 10, 11), both T1L and T3D had no transcriptase activities at temperatures of 62^{0}C and higher (Fig. 15, 62-70^{0}C controls). However, both T1L and T3D cores pre-heated to 62^{0}C for 15 minutes and then incubated at 48^{0}C for 1 hr still produced approximately 80% the amount of [^{32}P]UTP labelled material synthesized by cores maintained at 48^{0}C (Fig. 15, 16). Thus, the alteration(s) that prevent cores maintained at 62^{0}C from transcribing must be reversible. Cores pre-heated at 65^{0}C, which results in apparent removal of λ2 spikes from all particles and loss of genome from half the particles (Fig. 14D), and then returned to 48^{0}C still retain about 50% of transcriptase activity compared to cores maintained at 48^{0}C. In contrast, cores pre-heated at temperatures above 65^{0}C and then returned to 48^{0}C synthesized less than 50%, with transcriptase activity of cores heated at 70^{0}C undetectable (Fig. 15, 16).

3.10. The capacity of reovirus cores to produce *in vitro* transcripts correlates with presence of genome, not with presence of intact λ2 spike structures. It seems that T1L and T3D core particles have the same alterations when heated at high temperatures. Therefore, the following experiments were done mainly using T1L core particles. Ultrastructural

evaluations showed that the cores incubated at 65°C for 1 hr lost their spike structures (Fig. 14D). Surprisingly, these cores still retained as much as 50% transcriptase activity, compared to control cores (Fig. 15-16). This is a potentially novel system in which cores may be partially disassembled, but still possess transcriptase activity. These types of sub-core structures may prove useful for better delineating requirements of the RNA dependent RNA polymerase (RDRP) complex. To optimize generation of these spike-defective core structures, I performed kinetic studies. Cores were heated at 65 and 70°C for various times, then snap-cooled on ice and re-incubated at 48°C for 1 hour. They were then examined by electron microscopy and transcriptase activities determined. Spike structures were not visible after cores had been incubated at either temperature for 5 minutes (Fig. 17). Approximately 50% of the cores that had been treated at 65°C lost their genomes by 8 minutes of heat treatment, as judged by stain penetration. This value of 50% did not decline further with increased time of heat treatment (Fig. 17, 65°C and 14D). In contrast, 50% of the cores incubated at 70°C for 5 minutes retained their genomes, and this value declined to nearly 0% with increased time of heat treatment (Fig. 17, 70°C and 14E). Transcriptase activities of high temperature-treated cores paralleled the percentages of cores that still contained genomes, not the percentages of cores that possessed intact spikes structures at both tested temperatures (Fig. 17).

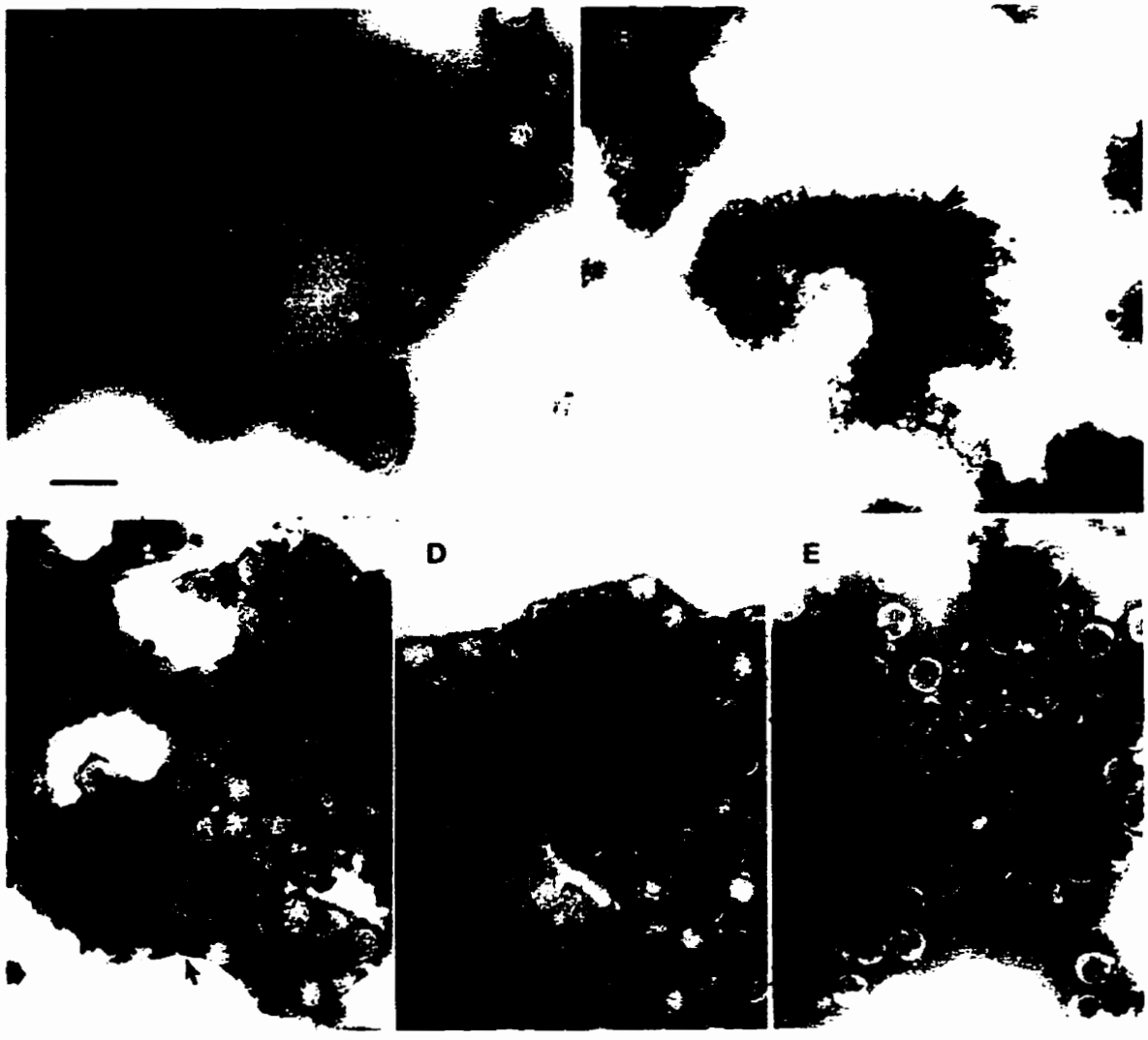

D
E

Fig. 14. Appearance of T1L cores after treatment at different temperatures. Core particles in transcription reaction buffer were incubated at 0^0C(A), 48^0C(B), 62^0C(C), 65^0C(D) and 70^0C(E) for 1h. Samples were negatively stained with phosphotunstic Acid and observed. All images shown are at a magnification of 100,000x; bar = 100nm. Arrow in (B) shows ssRNAs synthesized by cores. Arrow in (C) indicates an empty core which has lost its genome at high temperature. Arrow in (D) indicates the cavity at the expected position of a spike.

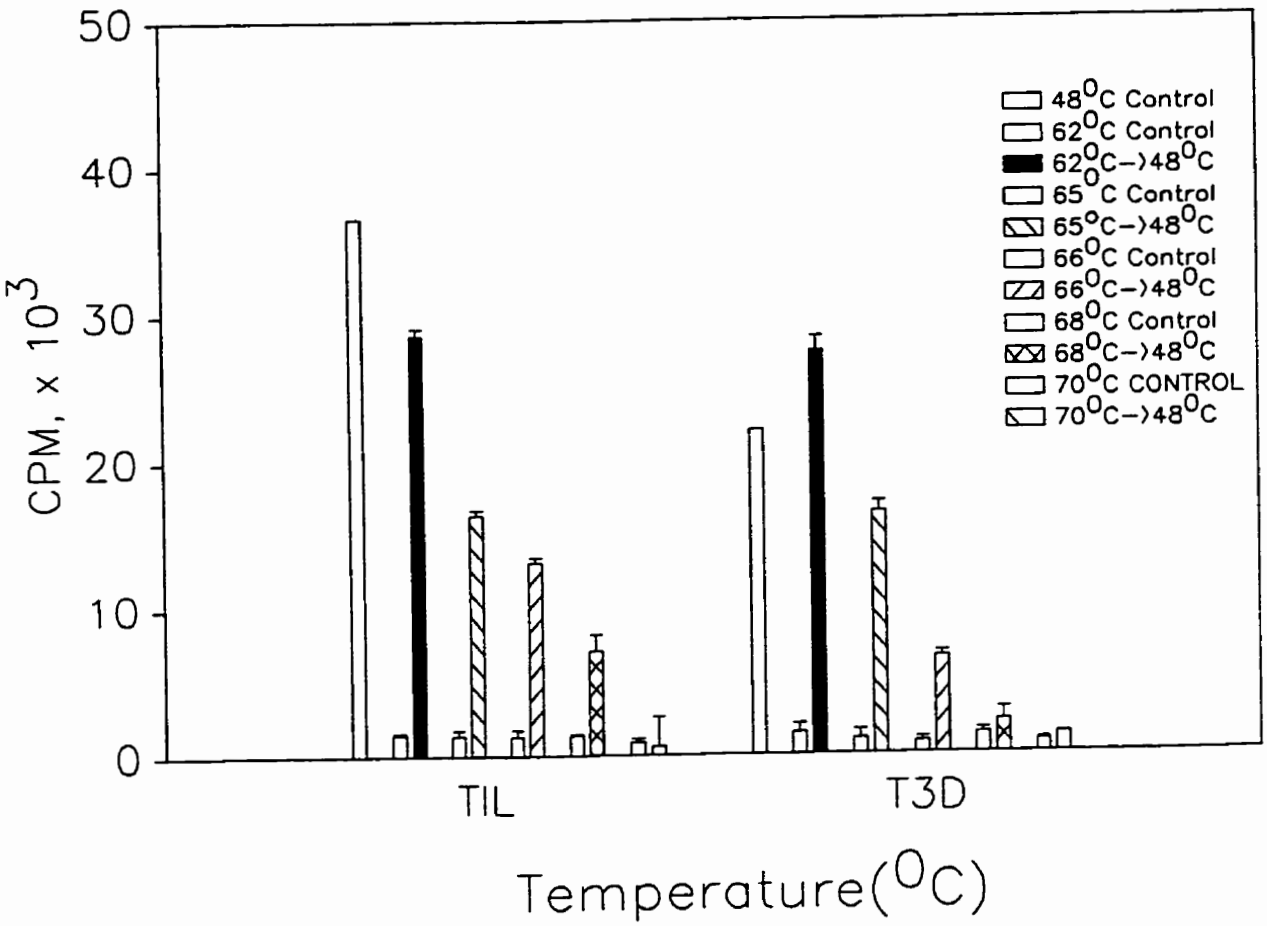

50
40
30
20
10
0
CPM, x 10^3
48^0C Control
62^0C Control
62^0C–>48^0C
65^0C Control
65^0C–>48^0C
66^0C Control
66^0C–>48^0C
68^0C Control
68^0C–>48^0C
70^0C CONTROL
70^0C–>48^0C
TIL
T3D
Temperature(^{0}C)

Fig. 15. Transcriptase assay at various high temperatures and at 48^{0}C of cores pre-heated at various high temperatures. In one set of reactions, a total of 3.5×10^{11} T1L or T3D core particles were incubated at indicated temperatures for 1 hour. The reactions were terminated by adding 10% TCA, and transcripts were precipitated onto filters and counted (controls, i.e. 48, 62^{0}C Control). In another set of experiments, a total of 3.5×10^{11} T1L or T3D core particles were briefly exposed at the indicated temperatures for 15 min, then chilled in an ice/water bath. Transcriptase assay components including 0.1 μCi [α-^{32}P]UTP were added (as detailed in Section 2.7). Aliquots were re-incubated at 48^{0}C for 1 hr, the reactions were terminated by adding 10% TCA, and transcripts were precipitated onto filters and counted. Activities produced by the cores pre-heated at high temperatures, then re-incubated at 48^{0}C (i.e 62^{0}C->48^{0}C) were compared to the activity produced by the cores maintained at 48^{0}C (100%). Results are the averages of four individual experiments. Error bars indicate variability (SD) between experiments.

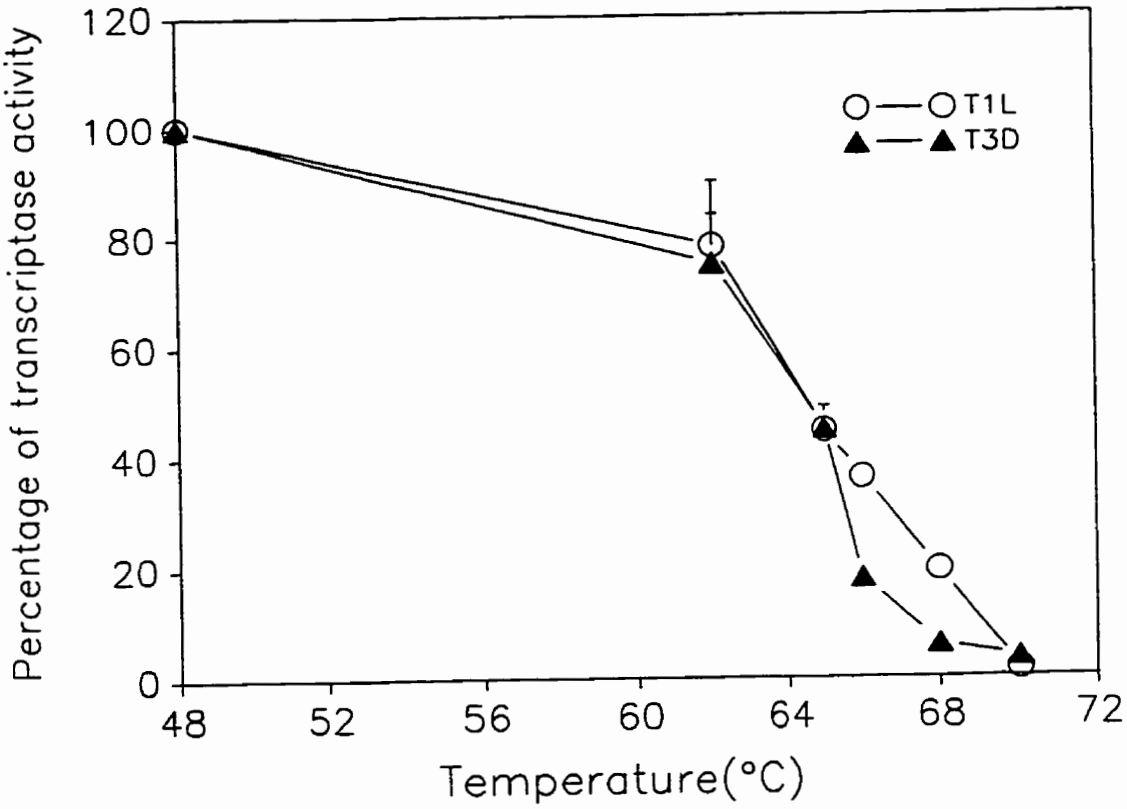

Percentage of transcriptase activity
120
100
80
60
40
20
0
48
52
56
60
64
68
72
Temperature(°C)
T1L
T3D

Fig. 16. Transcriptase assay at 48°C of cores pre-heated at various high temperatures. A total of 3.5×10^{11} T1L (○) or T3D (▲) core particles were briefly exposed at the indicated temperatures for 15 min, then chilled in an ice/water bath. Transcriptase assay components including 0.1 μCi [α-^{32}P]UTP were added (as detailed in Section 2.7). Aliquots were incubated at 48°C for 1 hr, the reactions were terminated by adding 10% TCA, and transcripts were precipitated onto filters and counted. Activities produced by the cores exposed to high temperatures were compared to the activity produced by the cores maintained at 48°C (100%). Results are the averages of four individual experiments. Error bars indicate variability (SD) between experiments.

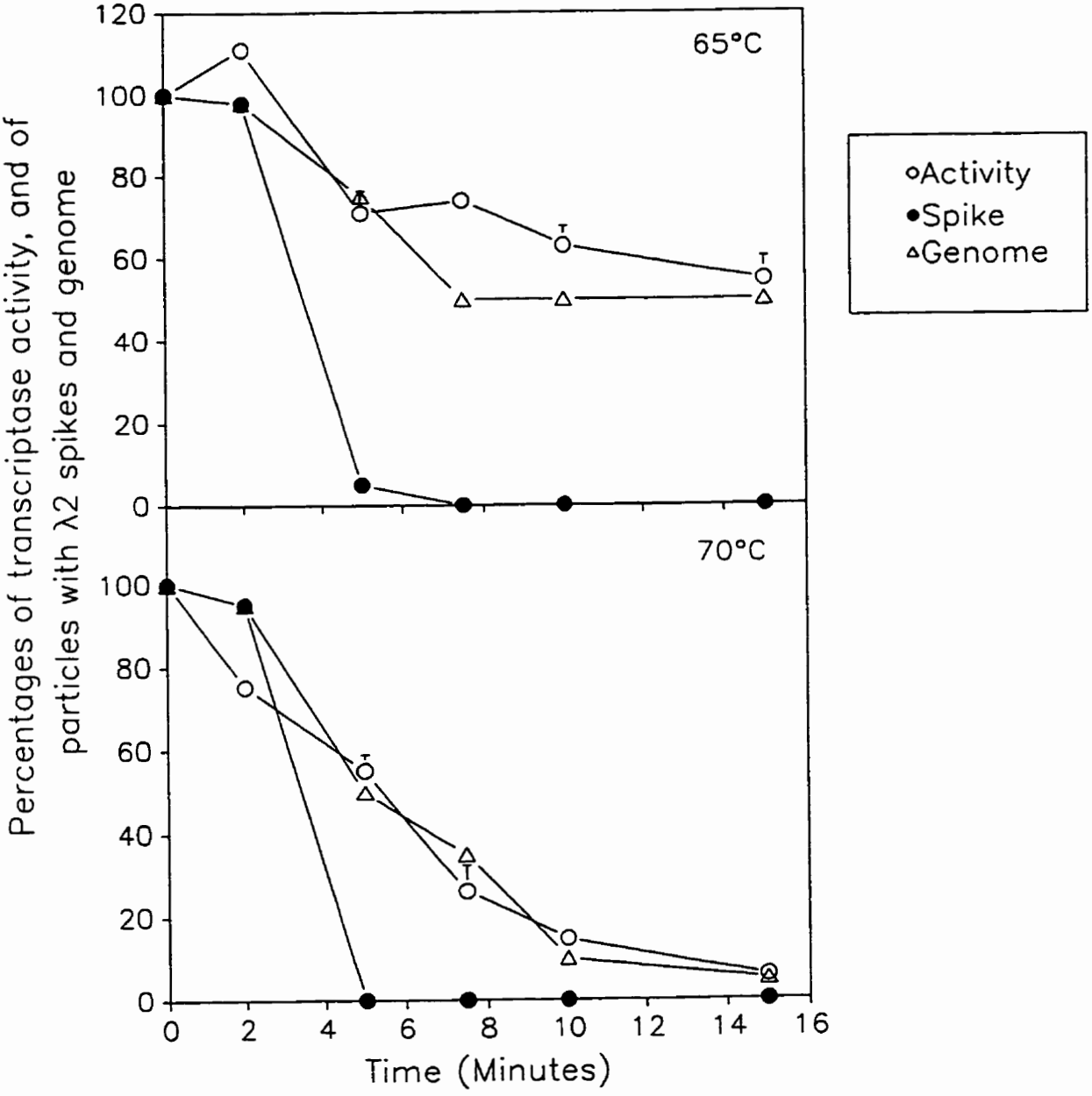

65°C
70°C
Activity
Spike
Genome
Percentages of transcriptase activity, and of particles with λ2 spikes and genome
Time (Minutes)
0
20
40
60
80
100
120
0
2
4
6
8
10
12
14
16

Fig. 17. Kinetics of reovirus transcriptase activity and core structure. T1L cores were incubated at 65°C or 70°C for indicated times, then transferred into ice/water bath where other transcriptase assay components were added. Aliquots were incubated either with 0.1 *μCi* [α-^{32}P]UTP (for *in vitro* transcriptase assay) or unlabelled (for EM) at 48°C for 1 hr. The transcriptase activities of the cores at indicated times were measured (○) as described in the legend of Fig. 16. The amounts of spikes (●) and genomes (△) within the cores were estimated by electron microscopy.

3.11. Radio-labelled products made *in vitro* by cores that contain genomes are viral ssRNA molecules. Reovirus contains a number of enzymatic activities which can convert ribonucleotides to acid-precipitable forms, such as poly(A) polymerase activity, alternative activities of the viral transcriptase which make initiator oligonucleotides (Stoltzfus *et al.*, 1974; Furuichi *et al.*, 1975). It has been shown that reovirus core particles can synthesize all 10 mRNA molecules at 37^{0}C *in vitro* (Skehel and Joklik, 1969; Hay and Joklik, 1971), and that elevated temperatures lead to an increase in the production of initiator oligonucleotides (Lai and Bellamy, 1971). Core particles pre-heated at 62 or 65^{0}C for 15 min still synthesized [^{32}P]UTP labelled material at 48^{0}C (Fig. 15, 16, 17). Therefore, to determine whether this labelled material is intact viral ssRNA or randomly-synthesized products made by the cores at different temperatures, I attempted to verify the nature of this material by hybridization assay. [^{32}P]UTP labelled transcripts synthesized by cores after various treatments were hybridized to unlabelled, purified T1L dsRNA. The RNase resistant hybrid molecules were resolved by 10% SDS-PAGE. For the cores incubated at 37^{0}C, the RNase resistant hybrid molecules were formed and clearly resolved in the gel (Fig. 18, lane 2), in agreement with previous reports (Skehel and Joklik, 1969; Hay and Joklik, 1971). The material made by the cores incubated at 42, 48^{0}C, and the cores briefly exposed to 65^{0}C for 15 min,

then re-incubated at 48°C for 1 hr also hybridized to genomic RNA (Fig. 18, lane 3-5). As expected, no hybrid molecules were formed by the cores exposed to 70°C (Fig. 18, lane 6), since almost no radioactive materials were detected at 70°C *in vitro* after re-incubation at 48°C for 1 hr (Fig 15, 16 and Fig. 17, 70°C). These results indicated that the transcripts produced are intact viral ssRNA.

3.12. The viral ssRNA molecules made *in vitro* by cores at different temperatures are functional. Hybridization assay had determined that core particles synthesized viral ssRNA molecules at temperatures ranging from 37°C to 48°C (Fig. 18, lane 2-4). In addition, cores pre-heated at temperatures up to 65°C were capable of synthesizing viral ssRNA when returned to 48°C. I then used *in vitro* translation assays (RRLS, Rabbit Reticulocyte Lysate System) to determine whether these ssRNA molecules were functional. Viral ssRNAs were generated as described above, the core particles removed and transcripts harvested as detailed in Section 2.10. The transcripts were then used to prime *in vitro* translation reactions. Translation reaction products were immunoprecipitated with anti-reovirus antiserum and proteins examined. All three size classes of viral proteins were made from ssRNA molecules synthesized by all temperature-treated cores except for cores incubated at 70°C which irreversibly lost their transcriptase activity to make ssRNAs (Fig. 19, lane 2-6). Similar protein

patterns were observed when pre-heated cores were re-incubated at 37°C instead of 48°C (data not shown). These results indicated that the ssRNA produced by the spike defective cores were authentic and fully functional viral mRNAs.

3.13. Purification of spike-defective core particles. The previous results indicated that brief heating (ie., 65°C > 5 minutes, or 70°C for 5 minutes) of reovirus cores appeared to produce a population of spike-deficient particles in which approximately half the particles still retain genomes and transcriptase capacity (Fig. 17). Such particles were then purified using CsCl gradients. Both types of treated, purified cores were examined by electron microscopy. The pentameric λ2 spikes were not visible on these cores (data not shown). However, when the samples were resolved by SDS-PAGE, the λ2 proteins were found in both pre-heated core samples (Fig. 20, lane 2-3). Due to the loss of genomes, and possibly protein λ2, the equivalence 1 OD_{260} = 4.2 X 10^{12} cores/*ml* would not accurately measure the quantities of heated cores. Therefore, to accurately determine relative concentration of various proteins within the different core preparation, I resolved a series of core samples at different concentrations in SDS-PAGE. Gels were scanned and number of particles scaled to each other by adjusting for the quantities of both major core proteins λ1 and σ2, based on the assumption that because there appeared to be no detectable change in the

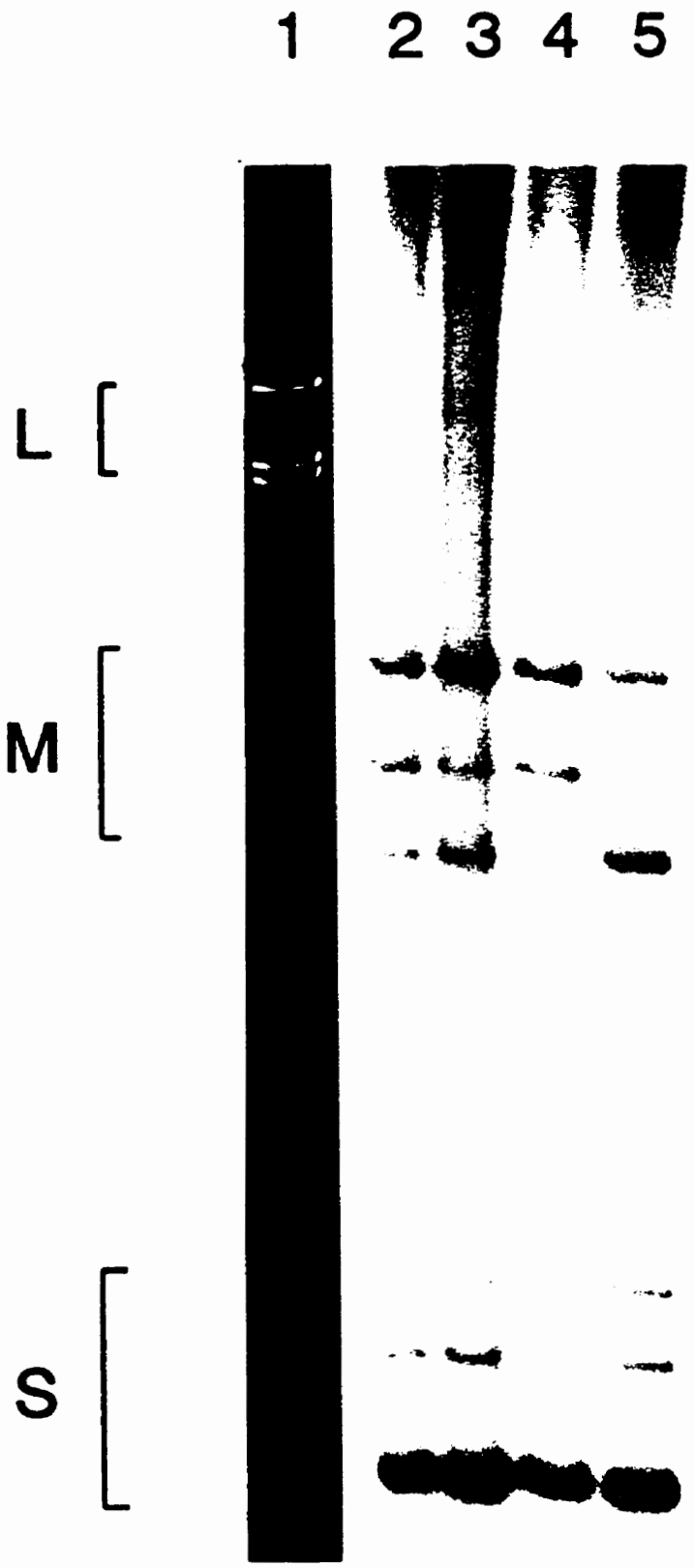

1 2 3 4 5 6
L
M
S

Fig. 18. Hybridization of ssRNAs made *in vitro* with cold dsRNA genome. 3.5×10^{11} gradient purified T1L cores in 50 μl transcriptase reaction buffer were either incubated at 37^0C (lane 2), 42^0C (lane 3), or 48^0C (lane 4) for 1 hr; or exposed to 65^0C (lane 5) or 70^0C for 15 min (lane 6), then re-incubated at 48^0C for 1 hr. After incubation with 0.5 μCi [^{32}P]UTP, the cores were pelleted (Beckman airfuge ® 26psi, 5 min). Single stranded RNAs in the supernatant were hybridized to T1L dsRNA as detailed in Section 2.10. The RNase-resistant hybrid molecules were resolved in 10% SDS-PAGE. Gels were fixed and autoradiographed. 40ng of unlabelled T1L dsRNA was resolved in the same gel and stained by Ethidium Bromide as marker (lane 1).

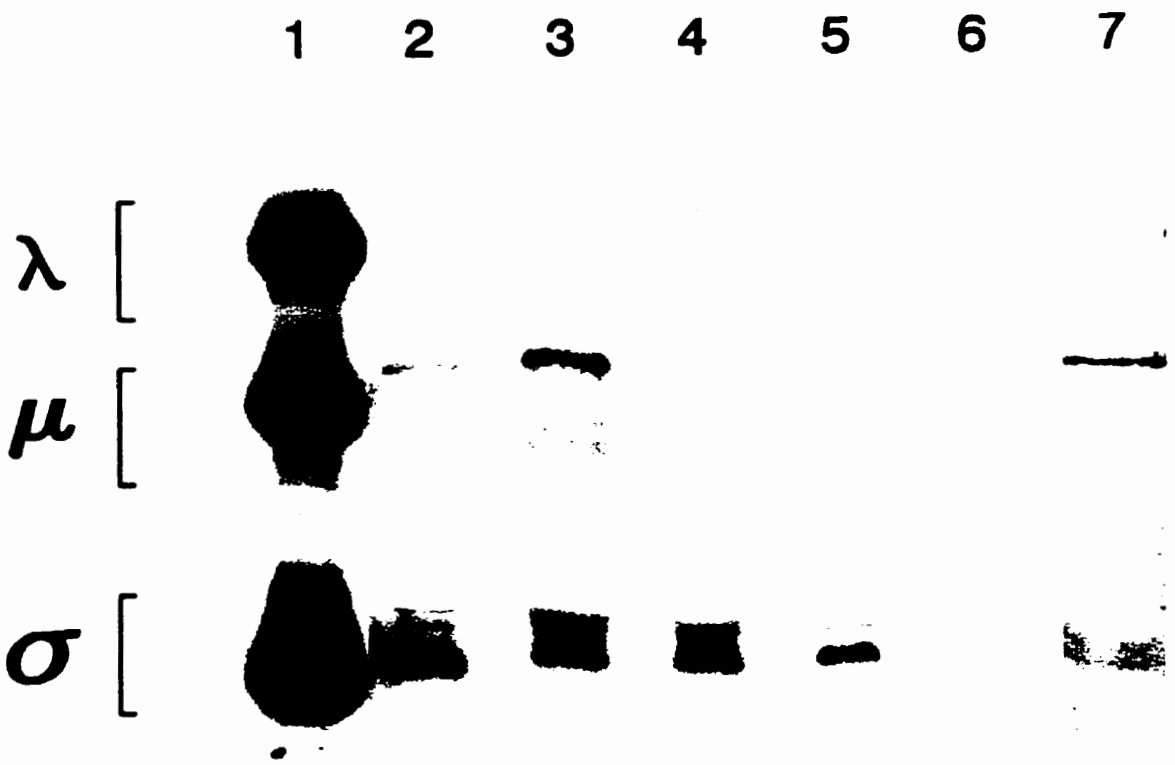

1
2
3
4
5
6
7
λ
μ
σ

Fig. 19. *In vitro* translation assay. Marker ^{35}S-labelled T1L viral peptides were resolved in the same gel (lane 1). Cores were incubated at 37^{0}C (lane 2), 42^{0}C (lane 3), or 48^{0}C (lane 4) only; or pre-heated for 15 min at 65^{0}C (lane 5) or 70^{0}C (lane 6), then re-incubated at 48^{0}C for 1 hr; or pre-heated at 65^{0}C, then digested with 100 *μg/ml* chymotrypsin at 37^{0}C for 30 min, digestions stopped by adding PMSF to 1 *mM*, and then treated particles re-incubated at 48^{0}C for 1 hr (lane 7). Core particles were pelleted as described in the legend to Fig. 18. Aliquots of 5 *μl* unlabelled ssRNAs in supernatant were added into 15 *μl* of the Methionine-deficient nuclease-treated Rabbit Reticulocyte Lysate System. Reactions at a final volume of 25*μl* were supplement with 10 units RNasin, labelled with 6.25 *μCi* ^{35}S-Methionine, then incubated at 30^{0}C for 1 h. Viral proteins were immunoprecipitated with anti-T3D reovirus-conjugated Protein A Sepharose beads and resolved in 5-15% SDS-PAGE. Gels were fixed and fluorographed as detailed in Section 2.12.

morphologies of heated core capsids (Fig. 14), these two major proteins could serve as internal references. After scaling, the λ2 bands of both heated cores were approximately 50% less intense than those of unheated cores (Fig. 20, Table 7).

The inability to observe pentameric spikes on heated cores by electron microscopy (Fig. 16, D-E) suggested that the λ2 spikes had been removed by the heat treatment. However, the apparently contradictory observation that λ2 was still present in these preparations (Fig 20, lane 2-3) suggests that it has undergone conformational changes and is either present on the core shell or that it is removed from the core capsid but may co-purify with the cores if trapped by the released genome. To examine these possibilities, I tested the sensitivity of the cores to chymotrypsin digestion. Native core particles are resistant to chymotrypsin treatment (Joklik, 1972); therefore, conformational changes in λ2, induced by high temperatures, might be revealed by increased sensitivity to chymotrypsin. Cores heated at temperatures 48^0C or lower remained insensitive to protease digestion (Fig. 21B, lane 0-3). By contrast, the amounts of λ1 and λ2 proteins in the cores exposed to 65 or 70^0C were greatly reduced (Fig. 21B, lane 4-5, Table 7). In addition, two extra protein bands between λ3 and μ2, possibly digested fragments of λ1 and λ2 from such treated cores were seen in the gel (Fig. 21B, lane 4-5). These observations indicated

that λ2 proteins in the cores heated at temperatures of 65 or 70^{0}C were sensitive to protease digestion and suggested that the pentameric λ2 spikes of the heated cores had undergone significant conformational alterations. Densitometric scans of these gels indicated that all of the λ2, and most of the λ1 proteins had been digested (Table 7). Electron microscopic observation of these treated cores indicated that over 50% of the 65^{0}C heated, protease-digested core structures still contained genomes.

3.14. Spike-deficient cores produce functional transcripts. The above results suggested that a combination of heat and protease digestion treatments could be exploited to generate spike-deficient core particles. Therefore, aliquots of 65^{0}C and 70^{0}C treated cores were briefly digested with chymotrypsin then purified in CsCl gradients. SDS-PAGE analysis of these purified particles revealed that less than 10% of the 65^{0}C heated, protease digested, gradient purified cores still contained genomes, while no genomes were found in 70^{0}C treated cores and that λ2 had been completely removed from both types of the heated and protease treated cores (Fig. 20, lane 4-5, Table 7). However, *in vitro* transcription assays of the gradient purified, 65 and 70^{0}C heated, protease-digested cores revealed virtually no transcript production (Table 8). The harsh conditions of CsCl gradient purification may have contributed to loss of activity (discussed in further detail

in Section 4), as previously reported in a related study (White and Zweerink, 1976). Thus, non-gradient-purified heated and protease digested particles were then used to prime *in vitro* translation assays. These translation assays showed that viral proteins were made from the spike-deficient cores that had been generated by a combination of 65^{0}C and protease digestion treatments (Fig. 19, lane 7), indicating that cores that lack λ2 have functional transcriptase activity.

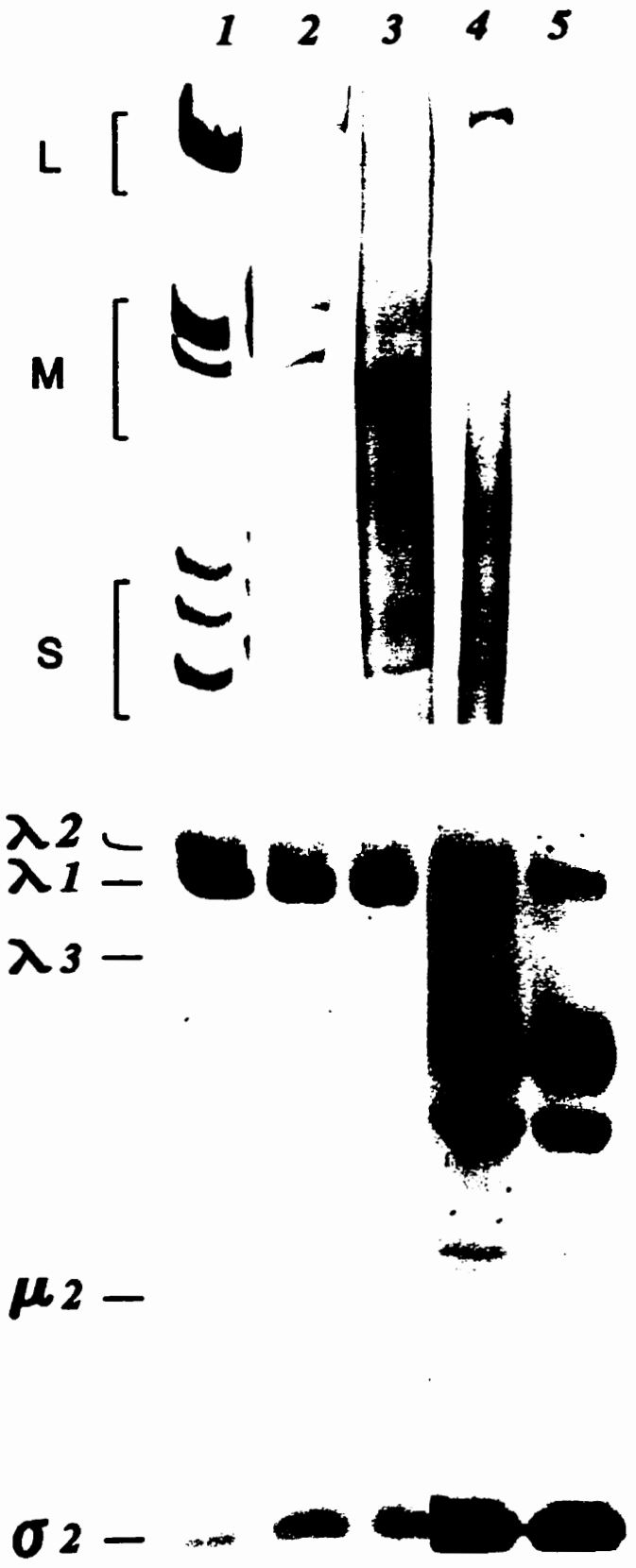

1
2
3
4
5
L
M
S
λ2
λ1
λ3
μ2
σ2

Fig. 20. Tris-Glycine-Urea (TGU) gel of CsCl purified core particles heated alone or treated with combinations of heat and protease digestion. Total of 3×10^{11} unheated T1L cores or a corresponding number of cores exposed to 65 or 70°C and purified by CsCl gradient, were resolved in TGU gels run at 8 mA for 15 hr. The upper part of the gels containing dsRNA genomes of the cores were cut and stained with silver staining. The protein contents of the cores in remaining part of the gels were stained with Coomassie Brilliant Blue. Unheated T1L core particles (lane 1); CsCl gradient purified cores exposed to 65°C (lane 2) or 70°C (lane 3) for 15 min; or 100 *μg/ml* chymotrypsin treated, pre-heated cores at 65°C (lane 4) or 70°C (lane 5) for 15 min. The gels were scanned with a LKB Ultroscan XL laser densitometer. Band intensities were analyzed in the Scanplot program and are presented in Table 7.

Table 7. Relative amounts of each protein in CsCl gradient purified cores either heated at high temperatures or treated with high temperatures and chymotrypsin[a]

Proteins	Mock	65°C	70°C	65°C+protease	70°C+protease
λ1	100	100	100	38.4±0.8	35.8±0.8
λ2	100	47.7±6	46.5±5.3	0	0
λ3	100	87.6±11.4	87.2±10.8	76.4±6.4	63.9±5.4
μ2	100	100±8.8	100±10	100±14	100±11
σ2	100	100	100	100	100

a: Gradient purified untreated cores (Mock) and cores either heated at indicated temperatures or treated with high temperatures and 100 μg/ml chymotrypsin were resolved in SDS-PAGE. Protein bands were scanned and analyzed as detailed in Section 2.12. The band intensities of treated cores were scaled to those of untreated cores, based on major core proteins λ1 and/or σ2 as detailed in the text. The results are the averages of five experiments ± SD. Values represent percentages of proteins found in untreated cores.

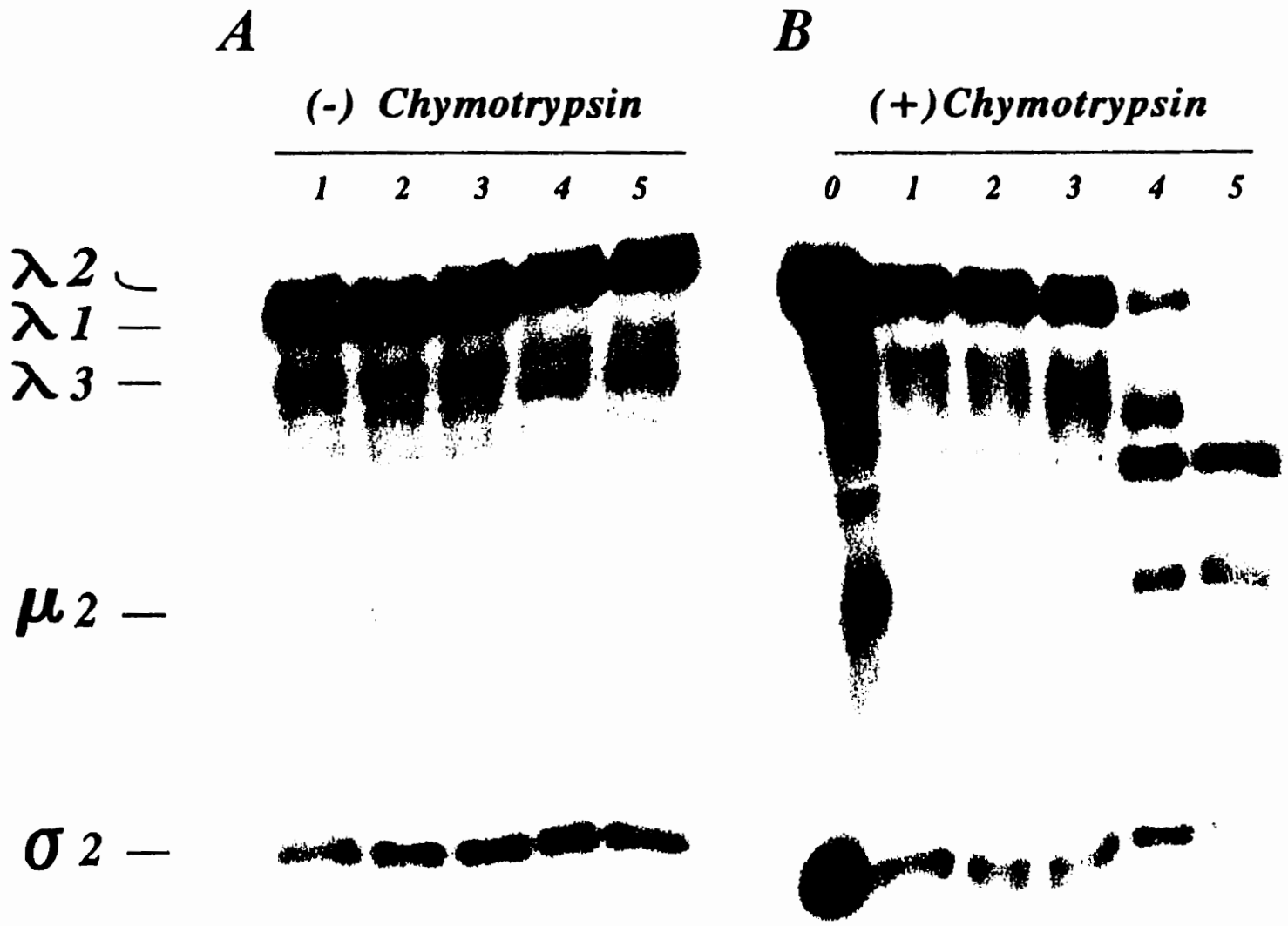

A
(-) Chymotrypsin
1 2 3 4 5
B
(+)Chymotrypsin
0 1 2 3 4 5
λ2
λ1
λ3
μ2
σ2

Fig. 21. Protease digestion of the heated cores. Gradient purified ^{35}S-Met labelled T1L cores heated at 0^{0}C (lane 0), 37^{0}C (lane 1), 42^{0}C (lane 2), 48^{0}C (lane 3), 65^{0}C (lane 4), or 70^{0}C (lane 5) were either mock treated (A), or treated with α-chymotrypsin at a final concentration of 100 *μg/ml* at 37^{0}C for 30 min (B). Resulting protein samples were resolved in TGU gels. Gels were fixed and fluorographed as detailed in Fig. 18 legend.

Table 8. Summary of genome contents, transcriptase activity and viral protein production in cores after various treatments[a]

		Before CsCl purification				After CsCl purification			
		(-) protease		(+) protease		(-) protease		(+) protease	
	Mock	65°C	70°C	65°C	70°C	65°C	70°C	65°C	70°C
Genome[b]	98	50	<1	50	<1	10	<1	10	<1
Transcriptase activity[c]	100	49.8±5	<1	42.5±4	<1	8.7±3	<1	7.1±1	<1
Viral protein production[d]	(+)	(+)	(-)	(+)	(-)	ND	ND	(-)	(-)

a: T1L core particles were heated at the indicated temperatures, treated (+) or not treated (-) with 100 μg/ml chymotrypsin, and examined either before or after CsCl purification. Values represent the percentages of indicated contents or activities as compared to non heated control (Mock) cores.

b: The proportion of cores that contain genome (as indicated by lack of stain penetration) was determined by electron microscopic observation as detailed in the text.

c: The relative amount of TCA precipitated ^{32}P-UTP material was determined as detailed in the text.

d: The ssRNAs synthesized by various cores were used to prime in vitro translation assays as detailed in Fig. 19 legend. Protein products were immunoprecipitated with anti-reovirus antibody and resolved in SDS-PAGE.

(+): reovirus proteins detected; (-): proteins not detected; ND: not determined.

3.15. Additional, preliminary results. During the course of my main project, structural and functional studies of reovirus RNA-dependent RNA polymerase complex, I also performed several additional experiments. Results from these additional experiments, such as partial sequencing of the T1L M1 gene and effects of actinomycin D on reovirus transcription are presented in this section. In addition, possible experiments which could follow these preliminary results in the future will be discussed in sections 5.1 - 5.4.

3.15.1. Sequence comparison of the T1L, T2J and T3D M1 gene encoding minor core protein μ2. This study indicated that minor core protein μ2 (encoded by the M1 gene) is the sole, or primary co-factor required by λ3, the reovirus RNA dependent RNA polymerase. Little is known about the function(s) of this minor protein. Furthermore, μ2 protein shares no similarity with other proteins in GenBank® (Wiener *et al.*, 1989). The M1 gene sequences of T1L and T3D have been determined. The amino acid sequence of μ2 were also deduced from the nucleotide sequence (Zou and Brown, 1992). Previous studies with other genes showed that T1L and T3D are closely related whereas T2J has less degree of homology with T1L and T3D (Cashdollar *et al.*, 1985; Wiener and Joklik, 1987, 1988, 1989; Dermody *et al.*, 1991; Seliger *et al.*, 1992). The relatedness of the genes among three reovirus serotypes are listed in Table 9. If the M1 gene has the same relatedness patterns as do other

genes (except the S1 gene), sequence comparison of the T2J M1 to those of T1L and T3D might narrow down the conserved regions among three serotypes. Therefore, it may be possible to locate homologous genes or proteins in data bases using smaller conserved sequences. In addition, the positions of conserved sequences may suggest functional domains of μ2. To better understand the structure and functions of μ2 protein and to study the relatedness pattern of the M1 gene among three reovirus serotypes, I attempted to sequence the T2J M1 gene. Oligonucleotide primers (M1-01, M1-07) complementary to both ends of the M1 gene were designed based on the known T1L M1 sequence and synthesized on a Beckman Oligo 1000 synthesizer as described in Section 2.13.1. The genomic dsRNA of T1L and T2J extracted from gradient-purified T1L and T2J viruses with phenol/chloroform was converted to cDNA by RT-PCR, then amplified as detailed in Section 2.13.2. Primer end-labelling and cycle sequencing of the T1L and T2J M1 genes were performed as described in Section 2.13.3. The partial sequence obtained from T1L M1 gene was identical to previous reported sequence (Zou and Brown, 1992). The partial sequence of T2J M1 at both ends (approximately 1 kb) (Table 10) was aligned with the same regions of T1L and T3D M1 sequences in the program, *ALIGN* (Genestream, France). A sequence homology of 62.9% among T2J and T1L or T3D was observed in this 1kb sequence (97% between T1L and T3D), suggesting the M1 gene may have similar relatedness pattern (less homology of T2J/T1L,T3D

vs T1L/T3D) as do other genes (Table 9). The partial amino acid sequence of T2J μ2 protein (encoded by the M1 gene) was deduced from T2J M1 gene sequence using the program, *Sequence Translation* (Virtual Genome Centre, U.Minnesota). The amino acid sequences of T1L and T2J μ2 proteins were also aligned in the program, *Align* as described above (Table 11). A sequence homology of 51.0% between T1L and T2J μ2 proteins was determined (Table 9). Primers corresponding to T1L M1 internal regions (about 1.3 kb) were also synthesized and used for T1L and T2J sequencing. Clear results were obtained from T1L M1 gene. However, no sequence could be obtained from T2J M1 gene. Furthermore, several primers designed based upon obtained partial T2J M1 gene sequence (as shown above) also failed to provide positive results. Alternative sequencing strategies will be discussed in Section 5.1.

3.15.2. The effects of actinomycin D on reovirus transcriptase activity. Inhibitors are commonly used to study enzymatic functions by blocking the specific activities of the enzymes. This strategy can be used in the structural and functional studies of reovirus RNA-dependent RNA polymerase (RDRP). Since there were no well-defined RDRP inhibitors available at the time these studies were initiated, actinomycin D, a known DNA dependent RNA polymerase inhibitor which binds to DNA and prevents movement of RNA polymerase, was used in this study. Previous studies demonstrated that

production of viral RNA in L cells was not inhibited by actinomycin D at a concentration of 0.5μg/ml (Kudo and Graham, 1965; Shatkin, 1965). Therefore, higher concentrations of actinomycin D were used to examine its effects on RDRP activity by *in vitro* transcriptase assays. The ssRNA synthesis was not inhibited by actinomycin D at a concentration of 5*μg/ml* for both T1L and T3D core particles (Fig. 22). Actinomycin D at a concentration of 20 *μg/ml* inhibited 50% of transcriptase activity of T3D cores (Fig. 24, ■). By contrast, the transcriptase activity of T1L core particles was not affected by actinomycin D at concentrations as high as 80 *μg/ml* (Fig. 22, □). These data suggest that actinomycin D can selectively inhibit RNA dependent RNA polymerase activity of at least one reovirus serotype. The responsible gene(s) and protein(s) for this serotypic difference could be determined using reassortant gene mapping method (see Section 1.2 and Sections 3.5-3.6) in the future as discussed in Section 5.4.

Table 9. The relatedness of the genes among three reovirus serotypes[a]

Gene/protein	1:3	2:1/2:3[b]
L1[c]	96%[d]	75%
λ3	98.3%	91%
M2	85%	76%
μ1	97.5%	97%
S1	5%	28%/9%
σ1/σNS	20%	20%
S2	86%	78%
σ2	99%	94%
S3	86.8%	78%
σNS	97%	86%
S4	94.6%	77.5%
σ3	97%	90%
M1	97%	62.9%[e]
μ2	98.6%	51.0%[f]

a: Only six of ten gene sequences of all three reovirus serotypes have been determined and compared as shown in this table.

b: T1L: 1; T2J: 2; T3D: 3.

c: The gene segment encodes following protein, such as, the L1 gene encodes λ3 protein.

d: The percent relatedness of the gene (upper valve), and deduced protein (lower valve), between two indicated serotypes.

e: The partial nucleotide sequence of T2J M1 gene determined in this study (Table 10) was used to align with the same regions of T1L and T3D M1 sequence in the program (ALIGN, Genestream, France) to obtain the degree of homology.

f: The deduced μ2 protein sequence of T2J was aligned with that of T1L (see Table 11).

Table 10. The partial sequence of T2J M1 gene[a]

1	GCUAUUCGCG GUCAUGGCUU ACAUCGCAGU UCCUGCGGUG GUGGAGUGGC
51	GUGCGAGUGA GGCUAUCGGG UUGCUGGAAU CGUUUGGUGU UACUGCCACG
101	GAGGACGAGA AUGAUGUUCA AUAUCAAGAU CAUGAUUAUG UGCUCGACCA
151	AUUGCAAUAU AUGUUGGAUG GAUAUGAAGC UGGAGAGGUA AUAGACGCGC
201	UAGUGUCUAG AAAUUGGUUA CAUAGAUCAG UACAUUGCCU GCUGACCGCA
251	GAAAGUCCAG UACUCGAGUA CUGGAAAAGU AAUCCGGCAG UCAUCCCAGA
301	GUCAGUUGAG CGCAGGUUUA GAAAGCGUCU AAUGAUGCAG AAAGAUCUGA
351	UGGAGGAUGA CGCCUACAAU CAAUUAGUCC GGCCUUUGAA CCUCUUCCAU
401	GUCUACUCUC CACUCGGUUC AUCCAUCCAA
1749	CGUGGCACAG GUCAAGGUAC CGUUCCAACG UCAAUCAGCU UCCAUUAUGC
1799	UUGACUAGUU AUGACGGACU UACGUGCCAC GUAAGUGAUG CAAUGCACAU
1849	GUGUCACGUA CUUGAACCUG UACUGAACAC UCGAAUUGCC AUGCCGAUAC
1899	UCACAGUGGU GCAAUCCAAG ACAUUGAUGC AAUGGUGUCG GUGUAGCGGA
1949	UUGCAUCACG UACGUUGUAC CGACGCCAAC UGUCUUAAGA GUUGCGUGAG
1999	AAAACUGGGU CAAUACCAUG CACAAGUAAU UGUAACUGAC AACACAGGUU
2049	GGGAGCCAGA CGUGUGCAUG CGAAGAGUGG UAAUCAAAGG GAAUCACAUG
2099	CCUUCGAAGU UGAUUUCCCG UUAUGCGUGC UUCUCUCUGA CUAUGAGAAU
2149	ACGAUGCACU GGACAUCGGG CAUUCCAUUG GGAGAGUUCA UCCUAUGGAG
2199	CGAGACUGGU AUUCCGAUCU UCCUUGCCGU GAUGUCGCGU CAUUAACGUC
2249	GAACCCCAUU UGGAAUGGGG GUAGGGGGCG GGCUAAGACU ACGUACGCGC
2299	UUCAUC

a: This partial sequence of T2J M1 determined as described in Section 2.13 was compared with the same regions of T1L and T3D M1 gene sequences in the program, *ALIGN* (Genestream, France). The degrees of homology among T2J and T1L or T3D are listed in Table 9.

Table 11. The alignment of the amino acid sequences of T1L and T2J μ2 protein[a]

```
            10         20         30         40         50         60
T1L MAYIAVPAVVDSRSSEAIGLLESFGVDAGADANDVSYQDHDYVLDQLQYMLDGYEAGDVI
    :::::::::::. :.:::::::::::::: :  : :::.:::::::::::::::::::::.::
T2J MAYIAVPAVVEWRASEAIGLLESFGVTATEDENDVQYQDHDYVLDQLQYMLDGYEAGEVI
            10         20         30         40         50         60

            70         80         90        100        110        120
T1L DALVHKNWLHHSVYCLLPPKSQLLEYWKSNPSVIPDNVDRRLRKRLMLKKDLRKDDEYNQ
    :::: .::::.::.:::  .: .:::::::::.:::..:.::.::::::..::: .:: :::
T2J DALVSRNWLHRSVHCLLTAESPVLEYWKSNPAVIPESVERRFRKRLMMQKDLMEDDAYNQ
            70         80         90        100        110        120

           130        140        150                   160        170
T1L LARAFKISDVYAPLISSTTATSAQPKWFGSLLRL-------LIC---PWLHMEKLIGEAD
    :.: ...  ::.:: ::      .. .   . : :         : :      .:: ...  .
T2J LVRPLNLFHVYSPLGSSIQWHRSRYRSNVNQLPLCLTSYDGLTCHVSDAMHMCHVLEPVL
           130        140        150        160        170        180

           180        190        200        210        220        230
T1L PASTSAEIGWHIPREQLMQDGWCGCEDGFIPYVSIRAPRLVMEELMEKNWGQYHAQVIVT
     .  .  :   .  . :::  :: : .:.     .:        .   .. ::::::::::
T2J NTRIAMPILTVVQSKTLMQ--WCRC-SGLH---HVRCTDANCLKSCVRKLGQYHAQVIVT
           190         200            210        220        230

            240        250        260        270           280
T1L DQLVVG-EPRRVSAKAVIKGNHLPVKLVSRFACFTLTAKYEMRLSC-GHST--GRGAAYN
    :.  .: ::     ..:::::::.: ::.::.:::.::    ::. : :: .   ....:.
T2J DN--TGWEPDVCMRRVVIKGNHMPSKLISRYACFSLT----MRIRCTGHRAFHWESSSYG
          240        250        260            270        280

     290
T1L ARLAFRSDLA
    :::.::::.:
T2J ARLVFRSSLP
          290
```

a: The partial amino acid sequence of T2J μ2 protein was deduced from T2J M1 gene sequence (see Table 10) using the program, *Sequence Translation* (Virtual Genome Centre, U.Minnesota). Then, it was compared with the same region of known T1L μ2 protein in the program, *Align* (Genestream, Grance). The relatedness ratio is listed in Table 9.

b: The four emboldened and underlined amino acids are the first four amino acids encoded by the second part of the M1 sequence (see Table 10).

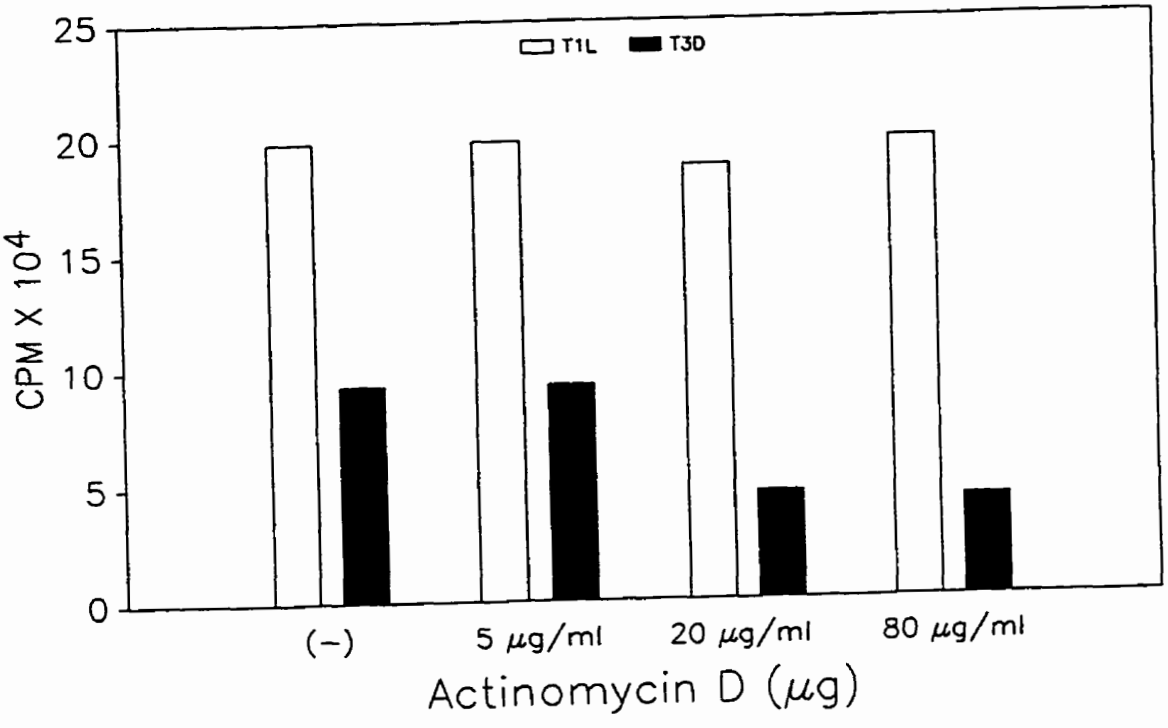

T1L
T3D
25
20
15
10
5
0
CPM X 10^4
(−)
5 μg/ml
20 μg/ml
80 μg/ml
Actinomycin D (μg)

Fig. 22. Effects of actinomycin D on reovirus transcriptase activity. Aliquots of 3.5×10^{11} gradient purified T1L (□) or T3D (■) core particles and other components of *in vitro* transcriptase assay (detailed in Section 2.7) were treated with different amount of actinomycin D ranging from 5 *μg/ml* to 80 *μg/ml* as marked. Then, the reactions were labelled with 0.1*μCi* ^{32}P-UTP and incubated at 50^{0}C for 1 h. The transcripts were precipitated with 5% TCA and collected onto filters. Radioctivities representing the amounts of synthesized ssRNAs by cores treated with actinomycin D at different concentations were measured as detailed in Section 2.7.

4. DISCUSSION

4.1. Role of minor core protein μ2 in reovirus transcription. Synthesis of progeny proteins is a necessary step in the replication of a virus that precedes assembly and release. Except for viruses whose genomes serve directly as message (*ie., Picornaviridae*) viruses must convert their genomic information into mRNA. For the *Reoviridae* the necessary enzyme(s) has RNA dependent RNA polymerase (transcriptase) activity. Reassortant gene mapping studies demonstrated that minor core protein λ3 (encoded by the L1 gene) determines the transcriptase pH optimum (Drayna and Fields, 1982). In addition, regions of protein λ3 share sequence homology including the GDD sequence motif with some other RNA polymerases (Morozov, 1989; Bruenn, 1991). Furthermore, recombinant studies showed recombinant λ3 has poly(C)-dependent poly(G) polymerase activity (Starnes and Joklik, 1993). These studies strongly suggest that minor core protein λ3 is the RNA dependent RNA polymerase (RDRP). However, by itself, recombinant λ3 is unable to synthesize transcripts from reovirus templates, including dsRNA, plus (capped or uncapped) and minus ssRNA templates (Starnes and Joklik, 1993). This suggests that other components of the core play important roles in transcription. This study indicates that there is a difference in both the temperature optimum of transcription and in the transcriptase efficiency

of purified T1L and T3D cores (Fig. 10, 11). Non-parametric and parametric statistical analyses of the temperature optimum reassortant mapping experiments indicated that serotypic differences mapped solely to the M1 gene (Table 4). Non-parametric and parametric statistical analyses of transcriptase efficiency reassortant mapping experiments indicated that serotypic differences mapped primarily to the M1 gene and the L1 gene (Table 6). A combination of the various statistical analyses indicated that most other genes were not implicated as playing any significant role in the transcriptase differences. The non-parametric analyses of each individual experiment suggested that the M2, S3, and S4 genes might be involved in determining serotypic differences in transcriptase temperature optimum (Table 4) and that the M3 and S1 genes might be involved in determining serotypic differences in transcriptase efficiency (Table 6). However, these apparent associations probably reflect non-random distribution of the gene segments among available reassortants; most reassortants with T1L L1 and M1 genes also possess T1L M2, M3, S1, S3, and/or S4 genes and most reassortants with T3D L1 and M1 genes also possess T3D M2, M3, S1, S3, and S4 genes. Indeed, the multiple regression analyses indicated that these additional genes did not contribute significantly to the effects of the M1 and L1 genes. Thus, various statistical analyses indicated that the reovirus M1 gene was the major determinant of transcriptase

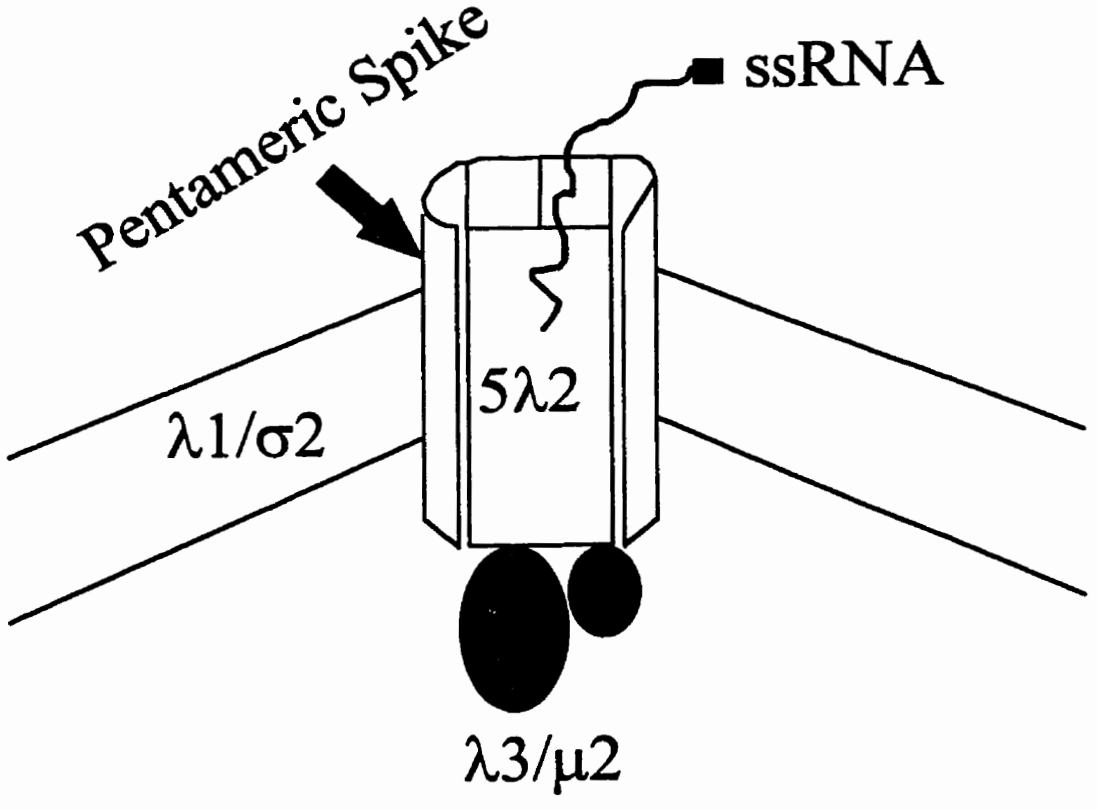
temperature optimum and the M1 gene and the L1 gene determined the transcriptase efficiency (Table 5, 6). In summary, these results indicated that the μ2 protein (encoded by the M1 gene) is involved in reovirus transcriptase activity and may be the co-factor (or primary co-factor) required by the λ3 protein, the RNA-dependent RNA polymerase.

The M1 gene is 2304 base pairs long (Wiener *et al.*, 1989; Zou and Brown, 1992) and encodes minor reovirus core protein μ2. Depending upon where translation of this gene's mRNA initiates, μ2 is predicted to be either 686 or 736 amino acids in length (Wiener *et al.*, 1989; Zou and Brown, 1992; Roner *et al.*, 1993). However, the most recent study suggested that translation of the reovirus M1 gene initiates from the first AUG codon to produce μ2 protein with 736 amino acids in both infected and transfected cells (Zou and Brown, 1996). There is about 98% amino acid identity between the T1L and T3D μ2 proteins (Zou and Brown, 1992), suggesting that the serotypic differences in transcriptase temperature optimum between T1L and T3D are attributed to the 2% variable sequences of T1L and T3D μ2 proteins. However, the protein shares no similarity with other proteins in GenBank® (Wiener *et al.*, 1989). Little is known about the function(s) of this minor protein. Reassortant mapping experiments suggest that μ2 plays a role in determining the level of virus growth in cardiac cells (Matoba *et al.*, 1991), in endothelial cells (Matoba *et al.*, 1993) and in Madin-Darby canine kidney (MDCK) epithelial cells

(Rodgers *et al.*, 1997). It is also involved in myocarditis (Sherry and Fields, 1989) and in organ-specific virulence in SCID mice (Haller *et al.*, 1995a). In addition, both λ1 and μ2 proteins were mapped as the determinants of nucleoside triphosphatase activities associated with cores (Noble and Nibert, 1997b). Furthermore, a study from our lab showed that a reovirus temperature sensitive mutant defective in protein μ2 failed to produce progeny double-stranded RNA (Coombs, 1996). Such observations led to the speculation that μ2 may play a role in RNA metabolism (Sherry and Fields, 1989; Matoba *et al.*, 1991, 1993).

Protein μ2 is present within the reovirus particle at approximately 12 copies per particle as is the other minor core protein λ3 (Nibert *et al.*, 1996). Because of the small copy number, which corresponds with the number of vertices on an icosahedral structure such as the core, it seems likely that both minor proteins may reside at or near the core vertices. Such a location would place these proteins near the pentameric λ2 spikes (60 copies/core) (discussed in more detail later in Section 4.3). Therefore, reovirus core proteins λ2, λ3, and μ2 may represent the structural and functional enzymatic complex that directs the transcription and capping of mRNA from the dsRNA genome. This hypothesis is supported by the recent detailed reassortant analyses of reovirus strains capable of inducing acute myocarditis (Sherry and Blum, 1994), as well as of reassortants capable of

inducing organ-specific virulence in SCID mice (Haller *et al.*, 1995b). The L1, L2 and M1 genes were mapped as major determinants in these studies which suggest the proteins (λ3, λ2 and μ2 proteins) encoded by these genes may interact. Cryoelectron microscopy and image reconstruction of the transcriptionally-active and inactive core particles showed that comformational changes were observed at positions underneath the spikes in the transcriptionally active cores (Yeager *et al.*, 1997). This observation strongly suggests the locations of minor core proteins λ3, μ2 and pentameric spike protein λ2 and possible association of these proteins. It also indirectly supports that these proteins play roles in reovirus transcription, since the conformational changes are only seen in transcriptionally active cores. The association between λ3 and μ2, and their roles in RNA synthesis were further confirmed by the investigations which demonstrated that both λ3 and μ2 regulated viral growth in mouse heart cells and Madin-Darby canine kidney epithelial cells (Sherry and Fields, 1989; Rodgers *et al.*, 1997). Significantly, the data from this study determined that both λ3 and μ2 are associated with serotype-dependent differences in transcription efficiency (Table 4), confirming the possible associations of minor core proteins λ3 and μ2. The above observations strongly support the concept that λ3, λ2 and μ2 proteins may form a structural and/or functional unit within the core particle (Fig. 23).



ds RNA Genome

Fig. 23. Proposed model of reovirus transcriptionally active core particle. In this model, only one of the twelve icosahedral vertices is shown in cross-section. The proteins and their locations in reovirus core particle are hypothesized in this model based upon this study and other studies as referenced in the text. Major core proteins λ1 and σ2 construct the architectural framework of the core. Each of twelve pentameric spikes protruding from the surface of the core is formed by five λ2 proteins (60 copies/core). The RNA-dependent RNA polymerase (RDRP) complex composed of each of two minor core proteins λ3 and μ2 (12 copies/core) may reside underneath the spike. The ssRNA molecules are transcribed from dsRNA genome within core particle by RDRP complex and released into cytoplasm through pentameric spike where they are modified. _____, represents ssRNA molecules; ■, is the symbol for the cap structure.

Collectively, these data suggest that proteins λ2, λ3, and μ2 may represent the structural and functional enzymatic complex that directs the transcription and capping of mRNA from the dsRNA genome. More detailed structural and functional analyses (extensively discussed in Section 5.1-5.4) may determine the precise locations of these minor core proteins and elucidate the roles played by these proteins in converting the genomic information of reovirus into mRNA.

4.2. Role of pentameric spike protein λ2 in reovirus transcription. The reovirus core is a macromolecular enzymatic complex comprised of multiple proteins and segments of dsRNA. Many of the enzymatic functions possessed by cores have not yet been assigned to individual core proteins. The purpose of this study was to define one of the enzymatic functions associated with the core, the reovirus RNA dependent RNA polymerase (transcriptase) activity. As discussed above (Section 4.1), this study and others hypothesized that minor core proteins λ3 and μ2, and pentameric spike protein λ2, may form a functional complex, performing transcriptase and capping activities. The relations and functions of λ3 and μ2 are extensively studied and defined (Section 4.1). However, the exact involvement of λ2 in this functional complex remains unanswered. Is λ2 protein involved in synthesis and modification of the ssRNA molecules or only involved in modification of the ssRNAs? One way to study functions of an

individual protein in such a complex is to remove the protein from the complex and study the effects caused by the absence of the protein. Alternatively, the roles of particular domains in a protein can be studied by similar deletional analysis. For example, these types of approaches have been successfully used to determine the roles of most structural proteins in the assembly of bacteriophage T4 (Black *et al.*, 1994), and the roles of different domains of the adenovirus E1A protein in binding to cellular proteins (Wang *et al.*, 1993). This study attempted to determine the functions of pentameric protein λ2 using similar strategy. Prior attempts to partially disassemble the reovirus core structure completely destroyed its enzymatic activities (White and Zweerink, 1976; Cashdollar, 1994), and made such subtractive analyses impossible to use for reovirus core functional studies. Spike-less cores have been previously generated; however, they lacked transcriptase activity (White and Zweerink, 1976). In addition, this lab has recently identified a reovirus temperature sensitive mutant that produces spike-less core particles at the non-permissive temperature (Hazelton and Coombs, 1997). The enzymatic activities of these mutant cores have not yet been determined. Thus, the data presented in this thesis shows, for the first time, partially disassembled reovirus core particles that still possess functional transcriptase activity.

4.2.1. Purification of spike-less transcriptionally active core particles. Electron microscopy indicated that heating cores to ≥ 65°C for ≥ 5 minutes caused a loss of all pentameric spike structures (Fig 14, Fig 17). However, the λ2 proteins remained detectable in these core samples after CsCl gradient purification (Fig. 20, lane 2-3). One possible explanation for this paradoxical observation is that the λ2 protein remains attached to cores but is denatured (unfolded) leading to disassembly of the spike structures. However, it seems unlikely that denaturation of approximately 700 kDa of protein (the aggregate molecular weight of five λ2 proteins) at each core vertex would go unnoticed. Another possibility is that the λ2 proteins are completely removed from the capsid structure by heat treatment but co-purify with the core particles if trapped by the escaped dsRNA genomes. This possibility is supported by the clear visualization of cavities at the core vertices (Fig. 14D, arrow) which suggests λ2 was completely removed, and by the ability to remove λ2 from gradient purified heated cores that had also been treated with proteases (Fig. 20, lane 4-5).

The core particles heated at 65°C alone or treated with heat and protease lost more genomic contents after CsCl gradient purification (10% vs 50% before purification) (Fig. 20, lane 2 and lane 4). The harsh condition of CsCl gradient may evoke this further leakage of the genomes. Since the transcriptase capacities of the spikeless cores depend upon

the genome contents that remain in these core particles (Fig. 17), the further loss of the genomes observed in these CsCl purified cores may demolish their transcriptase activities. Therefore, alternative purification methods using milder gradients such as rate zonal sucrose gradient were employed. The treated cores were loaded onto 10%-50% sucrose gradient in a Beckman SW41 rotor and centrifuged at 30,000rpm. These cores were pelleted in less than 15 minutes, whereas untreated cores usually form a band near the bottom of the gradient by 45 minutes under similar conditions. One possible explanation is that escaped viral genomes from the treated cores may facilitate the associations of these cores to generate a large complex which rapidly pelleted in the rate zonal sucrose gradient. The electron microscopic data which showed clump formations of treated cores (Fig. 14, Fig. 17) supported this possibility. Other types of gradients, such as isopynic renografin, also failed to purify these spikeless core particles with genomes.

4.2.2. Role of the pentameric spike protein λ2 in the production of functional reovirus transcripts. *In vitro* transcriptase assays of the spike-defective core particles generated by heat treatment at 65^{0}C indicated these structures can still synthesize radioactive material at 48^{0}C (Fig. 14-17), suggesting these spike-defective particles still retain transcriptase activities. Previous studies demonstrated that

reovirus core particles synthesize all 10 full-length mRNA molecules and initiator oligonucleotides at 30 to 37^{0}C *in vitro* (Skehel and Joklik, 1969; Hay and Joklik, 1971; Yamakawa *et al.*, 1981). In addition, elevated temperature is a factor which increases the production of these oligonucleotides (Lai and Bellamy, 1971). Therefore, it was necessary to verify that the product generated by the spike-defective cores at 48^{0}C after high temperature treatment was full-length mRNA. The hybridization and *in vitro* translation assays showed that both untreated cores and cores pre-heated to 65^{0}C synthesized full-length functional mRNA molecules (Fig. 18, 19). These radio-labelled ssRNA molecules were able to hybridize with genomic dsRNA and form RNase-resistant hybrid molecules which resolved by 10% SDS-PAGE. The three M and four S gene segments were well-separated. The L gene bands were weak and poorly separated (Fig. 18, lane 2-5). Previous studies have shown that the quantity of mRNA molecules of each species made *in vitro* and *in vivo* is inversely proportional to its molecular weight (Skehel and Joklik, 1969), which may explain why the L gene bands (Fig. 18, lane 2-5) and the λ protein bands from *in vitro* translation assays (Fig. 19, lane 2-5) are weak.

Kinetic studies that demonstrated a high correlation between proportion of cores that still contain genome and transcriptase activity, rather than between proportion of cores that possess visible λ2 spike structures and

transcriptase activity (Fig. 17), suggesting that the decline in enzymatic activity associated with high temperature (65-70^{0}C) treated cores is not caused by loss of the spikes. An alternative explanation is that this loss of enzymatic activity is caused by perturbation of the spike structure. However, several observations argue against this later possibility. Purification of heated cores in CsCl gradients led to a dramatic increase in the proportion of cores penetrated by Phosphotungstic Acid (from about 50% to > 90%), suggesting a further loss of genome (Table 8). This may be attributable to the relatively harsh conditions of CsCl purification. A similar conclusion was derived during other studies of spike-less core particles (White and Zweerink, 1976). These gradient purified cores had less than 10% the activity of untreated cores (Table 8), corresponding again with the percentages of genomes remaining within the cores. In addition, all the λ2 proteins and most of the λ1 proteins from 65^{0}C heated and protease treated cores that had not been gradient purified were digested (Fig. 21, Table 7), but these particles still possessed significant transcriptase activity and the transcripts appeared fully functional (Fig. 19, lane 7, Table 8). Thus, it appears that the capacities of the spike-less cores to produce functional mRNA transcripts are determined by the presence or absence of genomes, not by the presence or absence of λ2 spikes. Alternative purification methods (detailed in Section 4.2.2) have been tried in

attempts to purify spike-less cores that still contain genomes, but, to date, have been unsuccessful. These results indicated that the pentameric spike structures and the intact spike λ2 proteins are dispensable for the production of functional translatable reovirus mRNA molecules, and further suggest that λ2 proteins are not required for reovirus transcriptase activity.

4.3. Delineation of the roles of reovirus core proteins in the RDRP enzymatic complex. Transcription is an essential step in virus life cycle. In reovirus replication cycle, transcriptionally-active cores are produced from uncoating of the viruses. Then, ssRNA molecules are synthesized within core particles. This process of transcription and post-transcription modification requires enzymatic activities such as helicase, RDRP, RNA triphosphatase, guanylyltransferase and melthyltransferase (Fig. 24). As indicated earlier, the reovirus core particle is a multi-protein-ribonucleic acid complex that contains all components necessary for transcription, methylation, capping, and extrusion of progeny mRNA (Chang and Zweerink, 1971; Shatkin, 1974; Furuichi *et al.*, 1975), a process that is efficiently performed *in vitro* (Drayna and Fields, 1982; Yin *et al.*, 1996). Therefore, investigators in this field have been trying extensively to assign these enzymatic activities involved in transcription and post-transcription modification to individual core

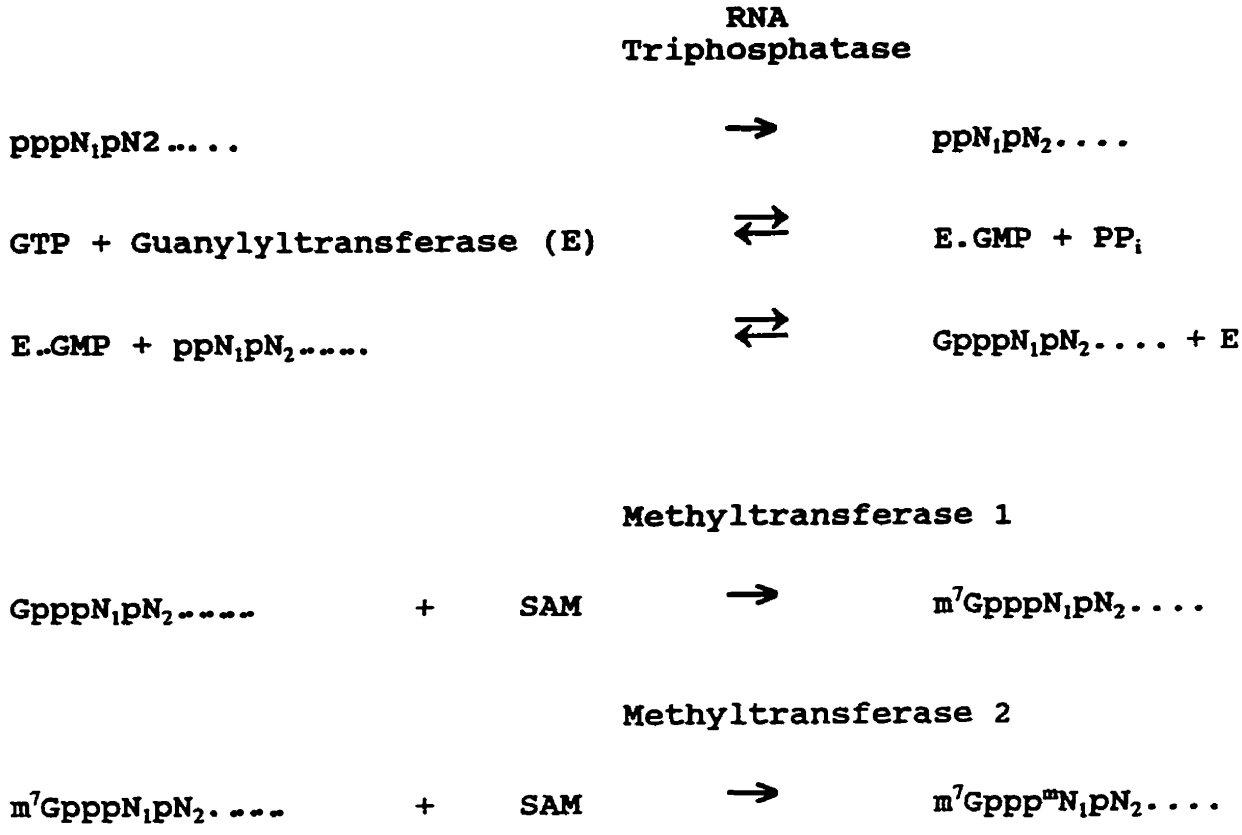


Fig. 24. The reactions that cap the 5' end of mRNA molecules. A guanosine residue methylated at N-7 position is linked to 5' end of mRNA via a unique 5'- 5' triphosphate bond to form a cap structure. In addition, the first nucleoside is also 2'-O methylated. $pppN_1pN2....$, mRNA molecule; N, nucleoside; SAM, S-adenosyl methionine.

proteins. It has been shown that core protein λ1 (encoded by the L3 gene) harbours two putative nucleotide-binding motifs [PRKTKGKS (A site) and DEAD (B site)] near its amino terminus and exhibits an affinity for double stranded RNA (Bartlett, J. A. and Joklik, 1988; Schiff *et al.*, 1988). Reassortant gene mapping analyses determined that λ1 carries a nucleoside triphosphate phosphohydrolase (ATPase) activity (Noble and Nibert, 1997a) which was later confirmed by recombinant studies using expressed λ1 protein in the yeast *Pichia pastoris* (Bisaillon *et al.*, 1997). Furthermore, it has been shown that recombinant λ1 protein can unwind double stranded DNA and RNA, suggesting a helicase activity possessed by this protein (Bisaillon *et al.*, 1997). These studies indicate that λ1 is responsible for unwinding of the reovirus dsRNA genome, an early step in transcription.

Reassortant gene mapping studies demonstrated that minor core protein λ3 (encoded by the L1 gene) determines the transcriptase pH optimum (Drayna and Fields, 1982). In addition, regions of protein λ3 share sequence homology including the GDD sequence motif with other known RNA polymerases (Morozov, 1989; Bruenn, 1991). Furthermore, recombinant studies showed recombinant λ3 has poly(C)-dependent poly(G) polymerase activity (Starnes and Joklik, 1993). These studies strongly suggest that minor core protein λ3 is the RNA dependent RNA polymerase (RDRP). However, by itself, recombinant λ3 is unable to synthesize transcripts

from reovirus templates, including dsRNA, plus (capped or uncapped) and minus ssRNA templates (Starnes and Joklik, 1993). This suggests that protein λ3 requires other components of the core for its accurate and efficient RDRP activity. This study determined that minor core protein μ2 (encoded by the M1 gene) was the sole determinant for transcriptase temperature optimum (Table 4); both minor proteins μ2 and λ3 controlled the transcriptase efficiency levels (Table 6). These data suggest that protein μ2 is also involved in reovirus transcription and it may be the co-factor required for λ3 protein for its RNA dependent RNA polymerase activity. These minor core proteins λ3 and μ2 possibly form a functional complex which is responsible for transcribing ssRNA from viral genome within core particles.

Biochemical studies showed λ2 proteins could form covalent λ2-GMP complexes when cores were incubated with [α-^{32}P]GTP, suggesting the λ2 spikes possess guanylyltransferase activity (Shatkin *et al.*, 1983; Cleveland *et al.*, 1986). Furthermore, vaccinia-expressed λ2 has all the enzymatic activities expected of the guanylyltransferase (Mao and Joklik, 1991). In addition, previous studies showed that protein λ2 is labelled when core particles are incubated with 8-azido-S-adenosyl[^{35}S]-Methionine (SAM) (Shuman and Hurwitz, 1980; Seliger *et al.*, 1987) (Fig. 23). Sequence analysis indicated that protein λ2 shares sequence homology with vaccinia virus 6.5S mRNA melthyltransferase (Seliger *et al.*,

1987; Koonin, 1993). These observations suggest that protein λ2 may also contain methyltransferase capacities, enzymatic activities necessary for the attachment of the methylated cap structure to the nascent mRNA transcripts (detailed in Section 1.4). It has been hypothesized that minor core protein λ3, the RNA dependent RNA polymerase (Drayna and Fields, 1982; Morozov, 1989; Bruenn, 1991; Starnes and Joklik, 1993), minor core protein μ2 and pentameric spike protein λ2 may form the structural and functional enzymatic complex that directs the transcription and modification of mRNA from the double-strand RNA genome (see Section 4.1) (Matoba *et al.*, 1991; Nibert *et al.*, 1991; Starnes and Joklik, 1993; Sherry and Blum, 1994; Yin *et al.*, 1996). With the exception of guanylyltransferase and possible methyltransferase activities, other roles, if any, that pentameric spike protein λ2 plays in the process of transcription were unclear. The work presented in this thesis demonstrated that transcriptase activity is retained by novel reovirus sub-core particles (spike-deficient core particles). These cores appear to have lost their spike structures and λ2 proteins but are still capable of synthesizing functional mRNA transcripts. These results suggest that pentameric spike λ2 proteins are not involved in the reovirus RNA-dependent RNA polymerase activity, but only involved in modifications (capping) of the nascent RNA molecules.

In conclusion, the roles of individual core proteins in

reovirus replication cycle, especially transcription process are further defined by this study. After entering cells, transcriptionally active core particles are produced from uncoating of the viruses. The double stranded RNA genomes are unwounded by core protein λ1. Then, the minus strands of unwound dsRNA genomes are transcribed by the transcriptase complex formed by minor core proteins λ3 and μ2 to synthesized ssRNA molecules within core particles. The newly-made ssRNAs escape to the cytoplasm through the pentameric spike channels (8nm) where pentameric spike λ2 proteins modify the 5' end of the nascent ssRNA molecules (capping) (Fig. 23, 24). More structure/function studies of the normal cores and spikeless cores could be performed in the future to determine the precise locations of the core proteins and to further elucidate the roles played by these proteins (see Section 5.1-5.4). For example, studies to examine guanylyltransferase activity and methyltransferase activity of spikeless cores, and cryoelectron microscopy and image reconstructions of the normal cores and these novel spike-deficient structures are discussed in more detail in Section 5.2-5.3.

5. FUTURE DIRECTIONS

5.1. Sequence comparison of the T1L, T2J and T3D M1 gene encoding minor core protein μ2. As shown in section 3.15.1, the partial sequence of T2J M1 at both ends (approximately 1 kb) was determined by end labelling cycle sequencing (Table 10). However, this method failed to determine the middle region of T2J M1 gene. Therefore, alternative sequencing methods such as a recombinant DNA sequencing method could be used in the future. Briefly, the M1 gene cDNAs could be converted from T2J dsRNA genome by RT-PCR using two primers containing BamH1 and HinD3 restriction cleavage sites and sequences complementary to both ends of the T1L M1 gene (see above). Then, the purified cDNAs could be cohesively ligated into pBluescript multiple cloning site and amplified. The white colones containing recombinant DNAs could be picked and plasmids could be purified then used in dideoxynucleotide cycle sequencing as discussed above. If the T2J M1 gene sequence could be determined in the future, the structures and functions of the M1 gene and μ2 protein can be further studied.

5.2. Structural and functional studies of the spikeless core particles. This study demonstrated that transcriptase activity is retained by novel reovirus sub-core particles (spikeless core particles) (see Section 3.14). Other

enzymatic capacities of the spikeless core particles, such as guanylyltransferase activity and methyltransferase activity can be determined in the future. A standard guanylyltransferase assay whose mechanism is illustrated in Fig. 23 could be used for this purpose. When cores are incubated with [α-^{32}P]GTP, the pentameric spike λ2 proteins with guanylyltransferase activity become radio-labelled by forming covalent λ2-GMP complexes shown in SDS-PAGE (Shatkin *et al.*, 1983; Cleveland *et al.*, 1986). The spikeless core particles could be generated as described in Section 3.13 and its guanylyltransferase capacity can be determined by above assay. Furthermore, core particles are also able to methylate the 5' end of reovirus mRNAs, the activity possibly carried by λ2 protein (Seliger *et al.*, 1987; Koonin, 1993). This enzymatic activity of core particles was determined by a standard methyltransferase assay. When core particles are incubated with [^{35}S]SAM {8-azido-S-adenosy[^{35}S]methionine} and ssRNA synthesized from cores in vitro, acid-precipitable radio-labelled material (methylated ssRNAs) will be detected (Fig. 24). This enzymatic property of the spikeless core particles could be examined by this assay. If the spikeless core particles lack above two enzymatic activities, these functions of the pentameric spike λ2 proteins would be confirmed. In addition, cryoelectron microscopy and image reconstructions could be also employed for structural and functional studies of the core in the future. For example,

the absence of the pentameric spike λ2 proteins in spikless core particles could be confirmed by this approach.

5.3. Structural and functional studies of reovirus transcriptase complex using expressed core proteins. Using various treatments and *in vitro* transcriptase assays, this study further defined the functions of the individual core proteins in reovirus transcriptase activity: μ2 protein is the co-factor of the reovirus RNA dependent RNA polymerase λ3; pentameric protein λ2 is not required for transcriptase activity. These observations can be confirmed and extended by additional studies in the future. For example, individual proteins or various combinations of complexes of λ2, λ3 or μ2 could be co-expressed for functional analyses. Previous studies indicated that the untranslated region at 5' of each gene determines the translation efficiency of the gene (Roner *et al.*, 1989). Moreover, the S1 gene encoding σ1 protein has the highest translational efficiency (Roner *et al.*, 1989). Therefore, DNA fragments encoding for λ2, λ3 and μ2 with different combinations (either all three genes, or only L1, L2; L1, M1), and 5' upstream regulatory sequences from the S1 gene could be ligated into a pECE vector suitable for expression in eukaryotic systems, and also capable of prokaryotic replication. Transfected L929 murine fibroblasts could be selected for plasmid-borne hygromycin resistance, and subsequently screened for in-frame expression of transfectant

proteins. Transfected, antibiotic-resistant cell populations could be subjected to limiting dilution, to clone or alternatively to isolated minimal viable populations. These populations could be carried in duplicate, and screened for expression of reovirus protein by a genetic-complementation technique. The transfected cells could be infected by reovirus strains which are temperature sensitive in the gene of interest. If successful infection is observed at nonpermissive temperature, this would indicate complementation, and therefore a successful transfectant. Secondary screening could then be performed on selected clones by immunochemical means, such as radioimmunoprecipitation and/or western blotting. Finally, the sequences of the successful transfectants could be confirmed and compared with wild-type gene to assess transfectant genotype. After isolation of three single-gene expression vectors corresponding to λ2, λ3 and μ2, the vectors could be amplified by prokaryotic passage to generate a large quantity of plasmid. The genes could then be isolated and ligated into a single vector for coexpression analysis (each gene possesses an independent ribosome binding sequence to facilitate independent expression, final plasmid size 13.6kb). Protein products isolated from the coexpression system could be assessed for molecular association, and their transcriptase activity. Protein association can be assessed by immuno-coprecipitation; cross-linking; or electron microscopy. The

RNA-dependent-RNA transcriptase activities of protein products with different combinations from coexpression could be assessed by *in vitro* transcriptase assays. The abilities of different protein products to make functional ssRNA can be examined by *in vitro* translation assay.

5.4. The effects of actinomycin D on reovirus transcriptase activity. It was shown that actinomycin D can selectively inhibit T3D but not T1L RNA dependent RNA polymerase activity (Fig. 22) (see Section 3.15.2). The responsible gene(s) and protein(s) for this serotypic difference could be determined using reassortant gene mapping method (see Section 1.2 and Section 3.5-3.6) in the future. The determined protein(s) responsible for this phenotypic difference between T1L and T3D must be involved in reovirus transcriptase activity. Therefore, the roles of individual viral proteins in reovirus transcriptase activity could be further defined. In addition, the effects of other inhibitors could be tested, and if serotypic differences exist, similar mapping experiments could be performed to determine which gene(s) are involved.

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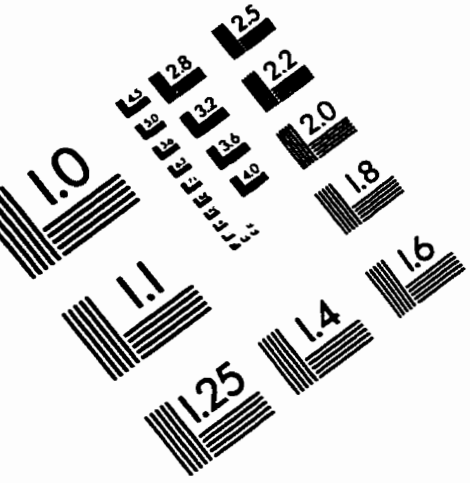

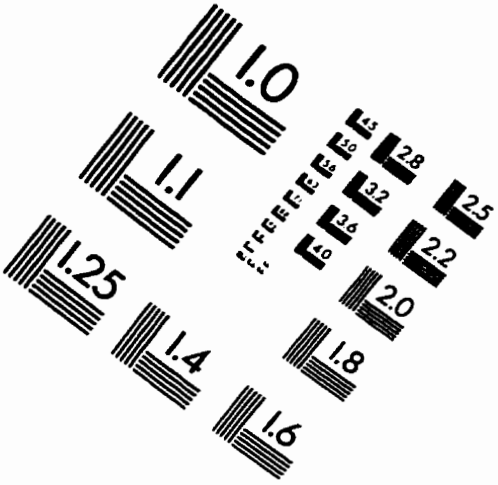

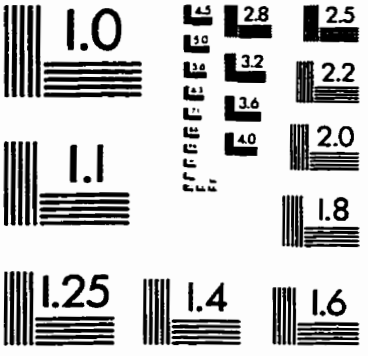

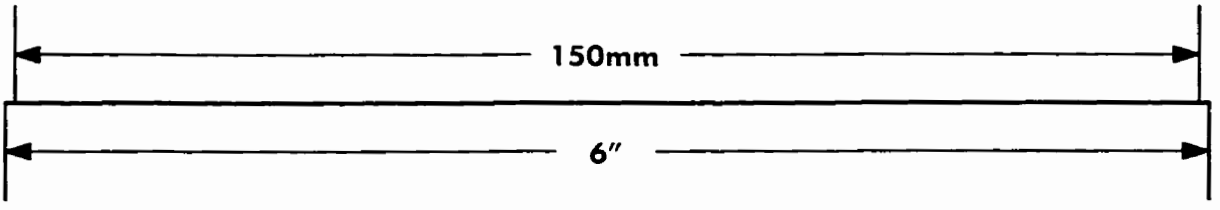

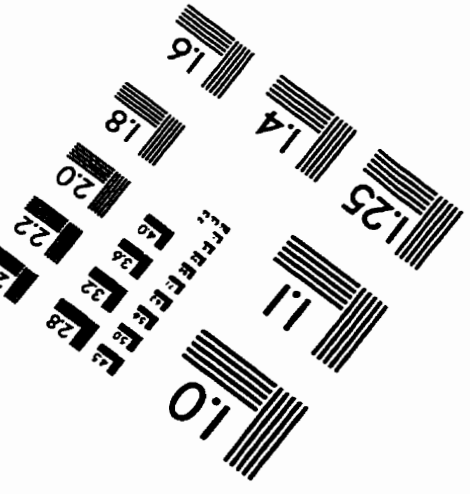

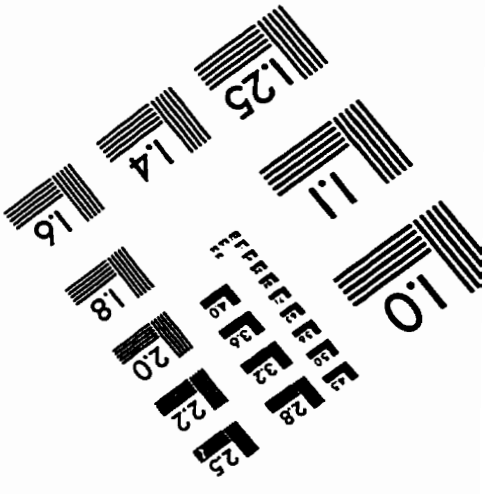

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