STUDIES OF THE REQUIREMENTS FOR THE INTRODUCTION OF SYNTHETIC GENES INTO REOVIRUS

A Thesis Submitted to the

School of Graduate Studies,

University of Ottawa

In Partial Fulfilment of the Requirements for the Degree

Of

Master of Science

Department of Microbiology and Immunology

Faculty of Medicine

By

Bogna Lasia

ISBN 0-612-15637-0

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

The mammalian reovirus genome is composed of 10 segments of dsRNA. They are transcribed and replicated by viral enzymes which are associated with the interior region of the core.

The purpose of this study was to introduce a synthetic reoviral gene into the reovirus particle. The gene was introduced in the form of (+) sense ssRNA transcripts and it was hypothesized that it would be recognized by viral replicase and encapsidated into nascent viral particles.

In attempts to introduce a synthetic gene into reovirus, reovirus type 1 infected cells were transfected with a synthetic deleted version of the T3 M1 gene of reovirus. Alternatively virions and cores modified by various chemical and physical treatments were incubated with synthetic (+) sense ssRNA copies of the M1 gene in replication assays. Neither of these methods resulted in successful replication of a synthetic gene or its introduction into reovirus. Studies examining the fate of transcripts showed that synthetic RNA was not recognized by viral proteins or particles when transfected into reovirus infected cells.

Efforts were made to select lethal M1 mutants by passage of T1, T3 and G2 reovirus in μ2 protein (product of M1 gene) expressing cells. These mutants could be used later as a selection system for reoviruses which rescued a synthetic M1 gene. In this system only reovirus with the rescued synthetic M1 gene should be able to grow on normal L929 cells. However, lethal M1 mutants were not obtained.

Reovirus particles that had been subjected to physical (sonication, heat, osmotic shock, cycle of freeze and thaw) and chemical (urea, formamide, pH 10.5, EDTA, DOC, guanidine, 2ME) treatments were analyzed by RNase A digestion and electron microscopy to examine structure and by transcription assay to relate structural changes to transcriptional activity. It was found that modified core particles could maintain or increase transcription activity relative to native cores. Their transcription activity had, in some cases, an altered temperature profile.

The most significant finding was the demonstration of active soluble transcriptase derived from viral cores that, previously, were thought to be the smallest component of the virus exhibiting transcriptase activity. This transcriptase complex is composed of dsRNA and λ3 protein. The problem of restricted access of exogenous RNA to the transcriptase can be overcome using this form of transcriptase and may be applicable to introduction of a synthetic gene into reovirus.

ACKNOWLEDGMENTS

I wish to thank my supervisor Dr. Earl G. Brown for his support, advice and encouragement throughout the period of this work.

I would also like to thank the members of my thesis advisory committee: Dr. Ken Dunock, Dr. Kathy Wright and Dr. John Bell.

Thanks must also go to Rod Nicholls for his expert technical assistance with electron microscopy.

Many thanks are due to the students and staff in the department for friendly working atmosphere and assistance on various occasions. I especially appreciate helpful discussions and technical expertise from Shimian Zou.

Special thanks go to my husband Peter to whom I dedicate this thesis. His technical help in writing this thesis, constant interest and unlimited patience and devotion allowed me to complete my study.

TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiv

1. INTRODUCTION 1

1.1 Reovirus particle morphology 1

1.1.1 Outer capsid structure 2

1.1.2 Inner core structure 6

1.1.3 Structure of the genome 7

1.2 Reoviral genome 8

1.3 Multiplication cycle of reovirus 9

1.3.1 Adsorption 10

1.3.2 Penetration 11

1.3.3 Transcription 12

1.3.4 Translation 14

1.3.5 Assembly 16

1.4 Reoviral pathogenesis studied with reovirus reassortment and anti-reovirus antibodies 18

1.4.1 Reovirus reassortment 18

1.4.2 Pathogenesis of reovirus infection 19

1.5 Physical and chemical stability of reovirus 20

1.6 Progress in the development of rescue systems for the introduction of cDNA genes into viruses 21

1.6.1 Minus strand virus expression systems 22

1.6.2 Development of expression systems for the Reoviridae 23

2. RATIONALE AND OBJECTIVES 26

3. MATERIALS AND METHODS 29

3.1 Bacterial culture and plasmid isolation 29

3.1.1 Mini preps 29

3.1.2 Midi preps 30

3.2 Methods used in plasmid constructions 30

3.2.1 PCR 30

3.2.2 Bacterial transformation 31

3.2.3 Colony hybridization 33

3.2.4 Restriction digestion 34

3.2.5 Agarose gel electrophoresis 34

3.2.6 Electroelution 35

3.3 In vitro transcription of plasmid DNA 35

3.4 Preparation of dsRNA from (-) and (+) sense ssRNA 37

3.5 Transfection 37

3.6 Preparation of the cytoplasmic extracts from eukaryotic cells 38

3.6.1 NP-40 based method 38

3.6.2 Triton X-100 based method 38

3.7 Reverse transcription of reovirus dsRNA from cytoplasmic extracts . . . 39

3.8 Radioimmunoprecipitation assay 40

3.9 Sucrose gradient centrifugation 41

3.10 Metabolic labeling of reovirus RNA in infected ceils 42

3.11 Northern blot hybridization 42

3.12 Viruses and cells 43

3.13 Growth and plaque assay of reovirus 43

3.14 Infection of suspension culture of L929 cells with reovirus and reovirus extraction 44

3.15 Preparation of cores 45

3.16 Chemical and mechanical treatment of cores and virus particles 45

3.17 CsCl gradient centrifugation of 4.8 M urea treated and sonicated cores 46

3.18 In vitro transcription/replication assay of reovirus activity 48

3.19 Polyacrylamide gel electrophoresis 49

3.19.1 4.5% urea polyacrylamide gel for separation of ssRNA 49

3.19.2 Protein polyacrylamide gel 49

3.20 Silver staining 50

3.21 Negative staining for electron microscopy 50

3.22 Scintillation counting 51

4. RESULTS 52

4.1 Introduction of the T7 promoter and Pst I restriction site into plasmids containing T1 and T3 M1 genome segments 52

4.2 Construction of a deleted T3M1 gene (M1D1) 54

4.3 Introduction of the synthetic deleted T3M1 gene into reovirus particle . 56

4.3.1 Introduction of M1D1 into reovirus infected cells by transfection 56

4.3.2 Attempts to detect the transfected gene in progeny virus 57

4.4 Examination of the fate of transfected M1 RNA in reovirus infected cells 62

4.4.1 Association of transfected synthetic RNA with viral proteins studied by radioimmunoprecipitation 62

4.4.2 Separation of the cytoplasmic components of reovirus infected cells transfected with synthetic viral RNA by sucrose gradient centrifugation 66

4.4.3 Analysis of reoviral ss and dsRNA by sucrose gradient centrifugation and radioimmunoprecipitation 71

4.4.4 Analysis of transfected M1D1 gene in sucrose gradient fractions by Northern blot hybridization 74

4.5 Selection of lethally mutated reovirus 76

4.6 Introduction of synthetic reoviral ssRNA into open-core particles 76

4.6.1 "Opening" reoviral particles and replication activity 78

4.6.2 Transcriptional activity of cores following different treatments . 83

4.6.3 Purification of cores treated with 4.8 M urea and 12 min. sonication 91

5. DISCUSSION 100

5.1 Introduction of a synthetic M1 gene into reovirus 100

5.2 Generation of a lethal M1 mutant 102

5.3 Studies of reovirus particle structure and transcription 104

6. CONCLUSIONS 108

7. REFERENCES 109

8. APPENDIX I 123

9. APPENDIX II 127

9.1 Selection of lethally mutated reovirus 127

9.1.1 Selection of the M1 mutant from the virus population by serial undiluted passage in μ2 expressing cells 127

9.1.2 Generation of M1 mutants by UV irradiation and passage in μ2 expressing cells 131

LIST OF TABLES

Table 1. Coding assignments of the 10 reovirus dsRNA segments, reovirus structural and nonstructural proteins and their characteristics. 4

Table 2. Summary of the effects of different treatments on reovirus structure and transcriptional activity. 90

Table 3. Analysis of T1, T3 and G2 titers following passage in normal L929 cells and μ2 expressing L929 and C127 cells 129

Table 4. Summary of plaques picked and assayed from different passages of T1, T3 and G2 virus. 130

Table 5. Isolation of a putative M1 mutant. 130

Table 6. Analysis of the effect of UV irradiation on reovirus infectivity. 132

Table 7. Determination of UV dose sufficient to abolish T1 and G2 infectivity. 133

LIST OF FIGURES

Fig. 1. Reovirus multiplication strategy and capsid structures. 5

Fig. 2. Schematic representation of physical and chemical treatments of cores and virions. 47

Fig. 3. Schematic representation of the cloning strategy for the T1 and T3 M1 gene for run-off transcription. 53

Fig. 4. Schematic representation of the M1D1 construction. 55

Fig. 5. Analysis of dsRNA from reovirus infected cells transfected with the synthetic M1D1 gene by 10% SDS PAGE. 58

Fig. 6. Northern blot hybridization of reoviral dsRNA to detect a transfected M1D1 gene. 60

Fig. 7. Agarose gel analysis of RT-PCR products from cell lysates transfected with M1D1. 61

Fig. 8. Analysis of the interactions of the transfected M1D1 gene with viral proteins by radioimmunoprecipitation. 64

Fig. 9. Interactions of transfected T1, T3 and M1D1 genes with viral proteins. 68

Fig. 10. Interactions of synthetic transfected M1T1 gene with viral components in infected cells after fractionation in 4-40% sucrose gradients. 69

Fig. 11. The position of synthetic M1D1 ssRNA in lysates of transfected cells, fractionated on 15-40% sucrose gradients. 70

Fig. 12. Analysis of reoviral ssRNA by sucrose gradient (15-40%) centrifugation and electrophoresis on 4.5% polyacrylamide gels. 72

Fig. 13. Analysis of reoviral dsRNA by sucrose gradient (15-40%) centrifugation and electrophoresis on 7.5% polyacrylamide gels. 73

Fig. 14. Analysis of M1D1 (+) ssRNA in transfected cell lysates 5 h post infection by 15-40% sucrose gradient centrifugation and electrophoresis on 4.5% polyacrylamide gel. 75

Fig. 15. Northern blot hybridization of transfected M1D1 (+) ssRNA to correlate its position with viral M1 ssRNA in sucrose gradient fractions. 77

Fig. 16. Characterization of treated reovirus particles by RNase A digestion. 79

Fig. 17. Electron micrographs of untreated and treated virus particles 81

Fig. 18. The effect of chemical and physical treatments of cores and virions on transcriptase activity at 34°C. 85

Fig. 19. Transcriptional activity of treated cores and virions at 50°C. 86

Fig. 20. Relative 50°C/34°C ratio of transcription 89

Fig. 21. Separation of treated cores in 1.3-1.6g/ml CsCl gradients 93

Fig 22. Protein and dsRNA content of CsCl gradient fractions of cores, sonicated dialyzed cores and cores treated with urea 94

Fig. 23. RNase A susceptibility of treated reovirus following CsCl gradient purification. 95

Fig. 24. Transcriptional activity at 34°C and 50°C of cores treated with 4.8 M urea and sonicated dialyzed cores before and after purification on CsCl gradients. 96

Fig. 25. Electron micrograph of transcriptase complex, "fraction #6". 97

Fig. 26. Electron micrographs of cores treated with 4.8 M urea and sonicated dialyzed cores following CsCl gradient centrifugation, both before and after subsequent sonication 99

Fig. 27. Schematic representation of the selection of the lethal M1 mutant by serial passage in μ2 expressing cells. 128

LIST OF ABBREVIATIONS

A	adenine
AA	amino acids
ab	antibody
AP	ammonium persulphate
cDNA	complementary DNA
bp	base pair
BSA	bovine serum albumin
C	cytosine
CIP	calf intestinal alkaline phosphatase
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleoside 5'-triphosphate
DTT	dithiothreitol
ds	double stranded
EDTA	ethylene diaminetetra acetic acid
EGF	epidermal growth factor
EM	electron microscopy
FBS	fetal bovine serum
G	guanine

h	hour
IPTG	isopropylthio-β-D-galactoside
ISVP	intermediate subviral particle
kD	kilodalton
2ME	2-mercaptoethanol
MEM	minimum essential medium
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MWCO	molecular weight cut off
NP-40	Nonidet P-40
nt	nucleotide
NTP	ribonucleoside 5'-triphosphate
O/N	over night
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
RIPA	radioimmunoprecipitation
RPM	revolutions per minute
RT	room temperature or reverse transcription

RT-PCR	reverse transcription followed by polymerase chain reaction
SDS	sodium dodecyl sulfate
sec.	second
ss	single stranded
T	thymine
T1	reovirus serotype 1
T3	reovirus serotype 3
TEMED	tetramethylethylenediamine
ts	temperature sensitive
U	uracil or unit
UV	ultra violet
vol.	volume

1. INTRODUCTION

1.1 Reovirus particle morphology

This thesis presents data on the introduction of synthetic reovirus genes into infected cells and also contains studies of the nature of transcriptional activity of reovirus particles. It is therefore necessary to introduce aspects of reovirus biology, structure and replication with an emphasis on structure and function relative to transcription and replication.

The mammalian reovirus is a member of the Orthoreovirus genus in the Reoviridae family. All members of the Reoviridae have a similar structure and multiplication strategy, contain 10-12 segments of dsRNA and enzymes required for transcription and replication (1).

The neutralization and hemagglutination-inhibition tests identified three basic serotypes of reovirus: type 1 Lang (T1), type 2 Jones (T2) and type 3 Dearing (T3). These serotypes can also be identified by different migration of their genome segments in polyacrylamide gels.

Reovirus particles possess two capsid shells which encapsidate 10 segments of dsRNA. Early studies established the presence of 8 structural proteins in reovirus particles (2) and their molecular weights (3). The presence of 10 reovirus translation products was demonstrated by comparison of polypeptides identified in the in vivo and in vitro systems (4). The protein products were assigned to genome segments (Table 1) (5). An 11th protein, σ1NS, was identified as the product of the bicistronic S1 gene (6). The rest of the genome segments are monocistronic, although several reoviral proteins are cleaved: μ1 , λ2 and μNS proteins (7).

1.1.1 Outer capsid structure

Ultrastructural studies of reovirus revealed that it has icosahedral symmetry (8). The μ1 (which is processed to μ1C/μ1N fragments that remain associated with particle), σ3 and σ1 proteins form the outer protein shell of the virion. The λ1, λ2, λ3, μ2 and σ2 proteins form the core, or inner shell (Fig. 1). The three outer capsid proteins σ1, and μ1 / μ1C and σ3 are modified in the conversion of virions to intermediate subviral particles (ISVPs) during uncoating in cells or as a result of chymotrypsin treatment. The conformation of the σ1 protein changes from compact to extended, μ1 /μ1C undergoes proteolytic cleavage and σ3 is removed. Further changes in the outer capsid proteins lead to the conversion of ISVPs to cores. ISVPs are infectious particles of reovirus, able to interact with cell surface receptors. Cores are non-infectious but transcriptionally active particles. Full uncoating is achieved when ISVPs are converted to cores which involves removal of σ1 and cleavage products of μ1, and conformational changes of λ2.

Sigma 1 protein. The σ1 protein (51 kD) is a receptor binding protein which forms a homotrimeric (9) or homotetrameric (10) fiber composed of a globular head (receptor binding domain) and fibrous tail (9). It contains two independent trimerization and folding domains: C and N terminals respectively. The compact structure of the head and tail of σ1 is separated by a stretch of polypeptide which has a more open, loose structure, which gives σ1 flexibility. The σ1 protein assumes a compact form in virions and an extended form in ISVPs (11, 12). The σ1 protein is located at the 12 vertices of the viral icosahedron. Monoclonal antibody studies revealed structural and functional relationships between σ1, μ1C and σ3 proteins. Removal of σ3 and/or proteolytic cleavage of the μ1C protein enhances the σ1 HA

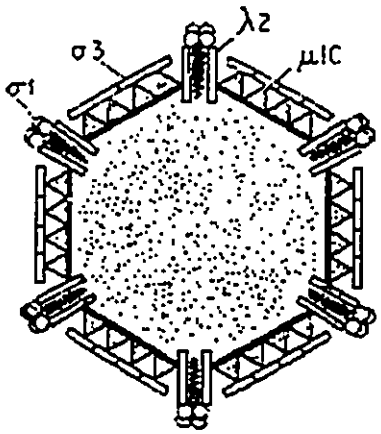
function (13). The number of σ1 fibers on reovirus particles is variable. The majority of reovirus particles possess 10, 9, 8 and 7 σ1 fibers. This phenomenon probably reflects limiting amounts of σ1 during reovirus assembly and the fact that only the complete lack of σ1 fibers renders the reovirus particle noninfectious (14).

Table 1. Coding assignments of the 10 reovirus dsRNA segments, reovirus structural and nonstructural proteins and their characteristics. (adapted from reference 15 and 32)

Particle(s)	Protein	Gene/ Size	Protein Mass (kDa)	Copies (virion)	Some functions and properties
Virion	σ3	S4 1196bp	41	600	Major outer capsid protein, hydrophilic, metalloprotein with Zn-finger motif
Virion, ISVP	μ1 (μ1C/ μ1N)	M2 2203bp	76	600	Major outer capsid protein, undergoes a series of proteolytic cleavages, myristoylated NH_2 terminus
Virion, ISVP, Core	σ1	S1 1416bp	51	36-48	Cell-attachment protein, viral hemagglutinin, neutralizing antigen, compact and extended conformations
Virion, ISVP, Core	λ2	L2 3916bp	144	60	Pentameric core spike, guanylyltransferase activity
Virion, ISVP, Core	λ1	L3 3896bp	137	120	Major core protein, binds genomic dsRNA, contains NTP binding motif, metalloprotein with Zn-finger motif
Virion, ISVP, Core	σ2	S2 1331bp	47	120-180	Major core protein, binds genomic dsRNA
Virion, ISVP, Core	λ3	L1 3854bp	142	12	Major core protein, putative catalytic subunit of viral polymerase
Virion, ISVP, Core	μ2	M1 2304bp	83	12	Minor core protein
Nonstructural	μNS	M3 2235bp	80	-	Binds ssRNA
Nonstructural	σNS	S3 1198bp	41	-	Anchor for viral assembly
Nonstructural	σ1NS	S1 1416bp	16	-	-

Fig. 1. Reovirus multiplication strategy and capsid structures. (I) Schematic representation of the outer capsid structure (taken from reference 32); (II) Changes in the outer capsid structure due to proteolysis, (A) virion, (B) ISVP, (C) core, (taken from reference 15), Bar 500 Å; (III) The reovirus multiplication cycle. Note that reoviral enzymatic activity is always associated with viral particles (taken from reference 106).

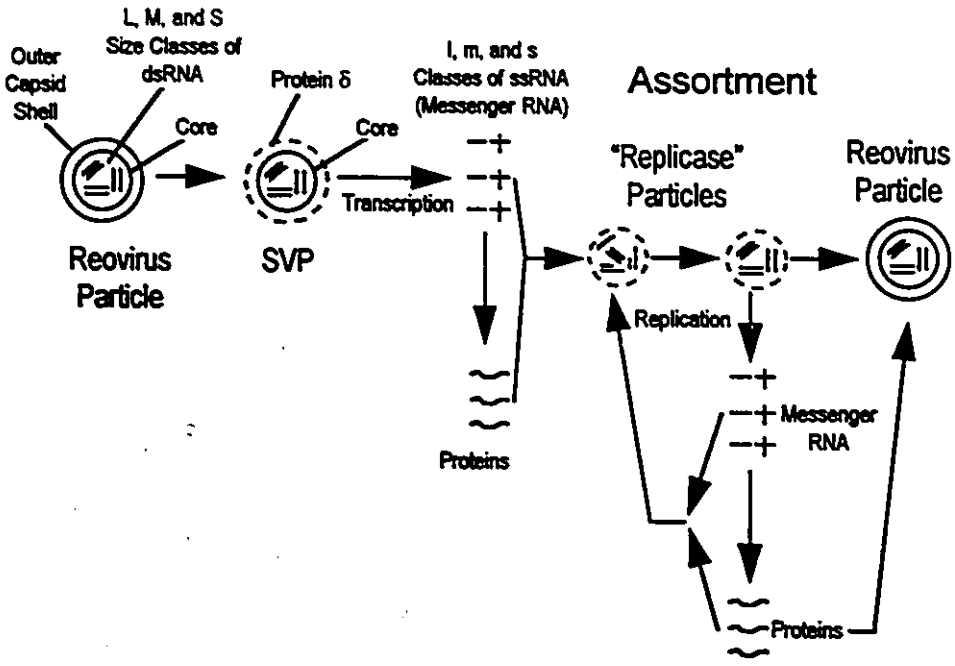
I


λ2
σ3
μ1C
σ1

II

A
B
C

III


L, M, and S
Size Classes of
dsRNA
Outer
Capsid
Shell
Core
Reovirus
Particle
Protein δ
Core
SVP
Transcription
I, m, and s
Classes of ssRNA
(Messenger RNA)
Proteins
Assortment
"Replicase"
Particles
Reovirus
Particle
Replication
Messenger
RNA
Proteins

Mu 1 protein. The μ1 protein (76 kD) is a major component of the outer capsid of reovirus virions and ISVPs and is present in 600 copies per particle. It forms trimers and lies between flower-like shapes on the virion surface (15). Protein μ1 undergoes cleavage events that probably occur during assembly and result in μ1C and μ1N. Both of these polypeptides together with μ1 are present on the surface of mature virions. During the conversion of virion to ISVP μ1/μ1C protein is proteolytically cleaved resulting in $\mu_{1\delta}$, δ and ϕ, which remain associated with the ISVP (16,17). The μ1 protein forms a complex, interlocking the outer capsid with other μ1 subunits, with σ3 protein and λ2 protein. The ethanol resistance phenotype of reovirus was mapped to the conserved amino acids within the δ cleavage product of μ1 protein which indicates that this outer shell protein is important for reovirus stability (18).

Sigma 3 protein. The σ3 protein (41 kD) is the second major component of the outer capsid shell. It has an ellipsoidal, finger-like shape and forms flower-like features on the surface of the virion capsid. It is present in 600 copies per particle and interacts with λ2 pentamers and the μ1/μ1C protein (15). The σ3 protein forms complexes with μ1 protein in vitro and its presence is required for μ1 cleavage (19, 20).

1.1.2 Inner core structure

The core is composed of five proteins: λ1, λ3, σ2, μ2 and λ2.

Lambda 2 protein. The λ2 protein (144kD) forms 12 pentameric core spikes. The λ2 pentamers undergo large conformational changes on conversion of ISVPs to core particles. During this process a central cavity is opened by rotation of the subunits and the spike is extended from 305 to 400 Å. Each of the three outer capsid proteins (σ1, σ3, μ1 and its

cleavage products) interacts with λ2, thus changes in contact with them caused by proteolytic digestion influence the conformation of λ2 spikes leading to their opening and extension.

Lambda 1 and sigma 2 proteins. The λ1 (137kD) and σ2 (47kD) proteins are the most abundant proteins in the core being present in 120 and 120 to 180 copies per virion respectively (15). The λ1 protein is proposed to be on the surface of the core, interacting with λ2 spikes (21) in the form of a dimer. The σ2 protein is proposed to occupy a more internal position than λ1 in the core. Both λ1 and σ2 bind reoviral dsRNA (22, 23). The dsRNA binding region in λ1 has been mapped to the 187 AA long amino-terminal region of the protein (23).

Lambda 3 and mu2 proteins. The λ3 protein (142 kD) and μ2 protein (83 kD) are minor components of the core, present in 12 copies per particle, which are probably associated with the 12 icosahedral vertices inside the core. Aside from their structural roles, capsid proteins λ2, λ3 and probably μ2 and λ1 have enzymatic functions (15).

1.1.3 Structure of the genome

The 10 segments of ds RNA comprising the reovirus genome are densely and orderly packed inside the core. The dsRNA strands are probably packed hexagonally with neighboring dsRNA running parallel to one another (24). Organization of reovirus dsRNA in the capsid is similar to DNA packaging in T4 and λ bacteriophage (25). Electron microscopic studies of extracted or released dsRNA from the core showed dsRNA in the form of "spiders" (26), "donuts" and dense "dots", indicating the presence of links between dsRNA segments (27). Reovirus cores also contain ss adenine rich oligonucleotides (28) which probably result from abortive RNA transcription (29).

The analysis of reovirus structure by cryoelectron microscopy and image reconstruction has allowed 27-32 Å resolution of protein and dsRNA structure. The reovirus genome occupies less than 240 Å of the approx. 600 Å diameter core. Addition of μ1C /μ1N and σ1 proteins gives ISVPs of approx. 800 Å in diameter and addition of σ3 gives virions of approx. 850 Å in diameter (15). Crystallization of the virus should permit more detailed characterization of reovirus structure, particularly the core structure. These studies have been initiated and hold promise for determination of the overall core structure at 8 Å resolution (30).

1.2 Reoviral genome

The reovirus genome is composed of ds RNA; the first dsRNA identified in nature. Another distinctive feature of the reoviral genome is its segmented character. The reovirus genome exists in the form of 10 genome segments of 3 size classes L, M and S (31). Recently all the genes of type 3 Dearing were cloned and sequenced. The sizes of the segments are shown in Table 1 (32). Sequencing data allowed the examination of reoviral genes for homology with other known sequences in gene banks, to search for functional motifs, to examine the mode of divergence of the reovirus serotypes and to predict the AA sequence of coded proteins (33) and their structure (34).

Initially, sequencing of the 60-90 residues at the 5' and 3' termini of 10 dsRNA segments identified a common tetranucleotide: GCUA at the 5' end and a pentanucleotide UCAUC at the 3' end of each (+) strand. These consensus sequences probably have roles as signals for transcription, replication or encapsidation. The 5' and 3' terminal regions of reovirus (+) strand RNAs also possess 22 sequences 20-30 nt long distributed among the 10

segments, of unknown significance, which exhibit either homology or symmetry (similarity in the same or opposite sense) with sequences in other genes.

Hairpins have been proposed to exist at the 3' end of the S1 gene and the 5' end of the M1 gene (35). Based on the comparison of 12 reovirus strains it was predicted that the 5' and 3' ends of message sense ((+) sense) S2 RNA form a long panhandle of duplex RNA (36). Studies of mutants containing deleted M1 genome segments identified the minimum essential sequence for M1 replication and assembly, which consists of 132-135 nucleotides from the 5' end and 183-185 nt from the 3' end of the plus strand. The M1 deletion fragment was predicted to form a stable secondary structure with the 5' and 3' termini forming panhandle and hairpin structures (37). The 59 nt region from the 5' end and 91 nt from the 3' end of the full length M1 gene are possibly associated together to form a secondary structural motif (38). All the segments of dsRNA contain a cap structure at the 5' termini of the (+) strand (39). Reovirus ss mRNA transcribed by parental cores contain blocked, methylated $m^7G(5')ppp(5')G^mpCp$ cap structures at the 5' end as well. The cap structure on reovirus mRNA and dsRNA provides protection against exonucleolytic attack and prevents formation of secondary structure involving the 5' end. The later effect would facilitate initiation of translation from mRNA and genome transcription from dsRNA (40, 41).

1.3 Multiplication cycle of reovirus

The multiplication cycle of reovirus is characterized by several distinctive features:

(i) Multiplication takes place in the cytoplasm.

(ii) Transcription and replication processes are catalyzed by viral enzymes.

(iii) The presence of free dsRNA is not detected during infection.

The dsRNA is always associated with protein complexes. It can be detected either in the parental, transcriptionally active cores or later, in nascent particles in which replication of (-) viral strand on the template of (+) sense mRNA is coupled with the assembly process.

The following section will describe the available data on reovirus adsorption, penetration, uncoating, transcription, replication, translation, assembly and release (Fig. 1).

1.3.1 Adsorption

Mammalian reoviruses have an extensive host range and are able to infect a variety of cell lines: fibroblasts, lymphoid cells, ependymal cells, CNS neurons and cultured pituitary cells among others (differs for type 1 and type 3 reovirus). Functional and biochemical characteristics of reovirus receptors indicate that binding of mammalian reovirus to cells involves sialic acid residues and that a growth regulator may also operate as a reovirus receptor (42). Indeed it was demonstrated that a region of the epidermal growth factor receptor, specifically the 105 kDa N-terminal cell surface domain of the EGF receptor, is the major protein recognized by reovirus (43, 44). Studies aimed at identifying other reovirus receptor proteins demonstrated 30 membrane proteins able to bind reovirus. Two of them ; 90 and 115 kD, were internalized during reovirus infection suggesting a reovirus receptor function (45).

Reoviral protein σ1 is responsible for binding to cellular receptors (46). The binding involves the C-terminal globular head of σ1 in a conformation dependent manner. The AA residues important for proper folding of the σ1 head have been mapped (47). Attachment of an intact virion to the cell receptor via σ1 protein is followed by receptor-mediated

endocytosis. Within the vacuolar endocytic vesicles that mature to lysosomes, the virion is proteolytically processed which allows for penetration into the cellular cytoplasm.

1.3.2 Penetration

Genetic and biochemical studies indicate that μ1 and its proteolytic products: μ1C, μ1N, $\mu_{1\delta}$, δ and ϕ mediate the penetration of the virus into the cytoplasm. Studies indicate that reovirus must be in the form of an ISVP to be able to mediate entry into the host cells. As described earlier, ISVP's are a partially uncoated form of reovirus, intermediate between infectious virions and transcriptionally active cores. Through conformational changes of σ1 and cleavage and conformational changes of μ1/μ1C, the ISVP is competent for attachment to cells and allows direct penetration of the virus into the cell. Conformational changes were demonstrated by the different susceptibility to pepsin of reovirus particles after binding to cell receptors and were particularly manifested by μ1C protein. It was also shown that these changes are reversible upon virus detachment (48). ISVPs were shown to be the only form of the reoviral particle that were able to bind to intestinal M cells (49), to induce formation of multisized channels in planar lipid membranes (50) and to cause membrane perturbations measured by ^{51}Cr release from mouse L cells. The chromium release function was then mapped to the cleavage products of the M2 gene (μ1 protein) (51). Other indications for the role of μ1 protein in penetration come from the finding that μ1N and $\mu_{1\delta}$ are myristoylated (16) and the prediction that the δ-ϕ cleavage junction is flanked by a pair of long amphipathic α helixes. Both myristoylation and amphipathic α helixes are well described structures permitting peptides to penetrate into the lipid bilayer of cell membranes and might act in

concert during reovirus penetration (17). Lastly, blockade in transmembrane transport has been mapped to the M1 gene (52).

1.3.3 Transcription

Transcription of reoviral ss mRNA can proceed once the outer protein shell is removed and virions are converted to cores. The core is a multifunctional enzymatic complex. Early studies of enzymatic activity demonstrated RNA transcriptase which acts on genomic dsRNA as a template (53, 54). Reovirus transcriptase is always associated with core structures and has never been obtained in soluble form (55).

As many as 12 strands of ssRNA of different lengths can be observed leaving core particles using electron microscopy (56). This observation combined with cryoelectron microscopy and image reconstruction (15) showing dramatic conformational changes of λ2 protein during ISVP to core transition led to the prediction that newly synthesized mRNA exits the core through λ2 channels.

The putative viral transcriptase, λ3 protein, has never been purified in a soluble active form (57). The experimental data in favor of the λ3 protein being the reoviral transcriptase are as follows:

(i) λ3 protein contains blocks of amino acids that are conserved for all RNA polymerases (58, 59);

(ii) λ3 controls the pH optimum of the transcriptase (60);

(iii) when cloned into the TK gene of vaccinia virus strain WR under the control of T7 polymerase promoter λ3 is able to transcribe poly (C) in vitro (61).

The M1 gene encoding the μ2 protein was found to control the difference in temperature optimum for transcription of type 1 and type 3 reovirus implying its role in reoviral transcription as well (62). It is tempting to speculate that λ3 and μ2 form a protein complex since both of them are present in 12 copies per core and both may be associated with the 12 icosahedral vertices; the site where nascent mRNA is extruded.

The λ2 protein has a function in reoviral transcription since its removal from the core results in inhibition of transcription (21). It was shown that λ2 protein exhibits guanylytransferase activity and takes part in cap formation on the 5' end of nascent transcripts (63). Photochemical crosslinking experiments have shown conformational changes of transcriptionally active cores probably induced by ribonucleoside triphosphates. These studies proposed that λ1 (product of L3) has transcriptase activity (64) although other studies have assigned only a structural function to λ1 within reoviral cores (22, 23).

Intact virions were shown to be transcriptionally active but were not able to elongate nascent ssRNA so that only short capped oligonucleotides corresponding to the 5' end of viral mRNA were detected (54, 65). It seems that conformational and space constraints related to the presence of the outer shell proteins on the virion (rather than free access of reaction mixture ingredients and activation of the transcriptase) play a role in preventing transcriptase from synthesizing full-length transcripts.

The mechanism of reovirus transcription is analogous to the transcription of DNA; it is conservative with respect to the template. Transcription is also asymmetric with only the (-) strand of dsRNA serving as a template. The products of the reaction are full-length ss copies of the 10 ds genome segments. The transcription reaction has an absolute requirement

for magnesium, its pH optimum is between 7.8 and 8.2 and its rate of synthesis increases with temperature up to 50°C (66). The rate of nucleotide incorporation for reovirus transcriptase is estimated to be within the range of 2-60 nucleotides/sec. (66, 67, 68).

At early times of transcription, the rate of transcription for different size classes of ssRNA is S>M>L (67). This observation is in accord with the model in which each segment is transcribed simultaneously and independently of the others with a constant rate of synthesis. In this event the frequency of transcription would be inversely proportional to the molecular weight of the segment. EM studies with "active cores" support this mechanism as well (56). However, it was also found that in the presence of cycloheximide (an inhibitor of protein synthesis) only L1, M3, S3 and S4 are transcribed in cells infected with wild-type or ts mutant reovirus. These transcripts were shown to be functional mRNA. A model was proposed in which "pre-early mRNA" regulates virus or cell-coded repressors and is involved in turning on the transcription of the late segments (69, 70).

1.3.4 Translation

The mRNA made during reoviral transcription serves two purposes: it is used by the cellular translation machinery to produce viral proteins and serves as a template for nascent dsRNA replication. Translation of reoviral proteins in tissue culture is extensively controlled (71). L1 (λ3), M1 (μ2) and S1 (σ1) are translated very inefficiently , L2 (λ2), L3 (λ1) and S2 (σ2) are translated with intermediate efficiency and S4 (σ3), M2 (μ1), M3 (μNS) and S3 (σNS) are translated efficiently (71, 72). L1 and S1 mRNA possess a "weak" initiation codon, whereas the rest of the reoviral mRNAs possess "strong" initiation codons as defined by Kozak (73, 74).

The S1 mRNA is of particular interest because it is translated into two proteins σ1 and σ1NS. It was found that regions in the vicinity of the initiation codon as well as length and secondary structure of the 5' untranslated region can influence translational frequency (72). It was also determined that the region between ORF1 (AUG 14) and ORF2 (AUG 75) plays a major role in determining the relative efficiency of σ1 and σ1NS synthesis (74). The analysis of steady-state distribution of translating ribosomes on S1 mRNA revealed ribosome pausing and a less even distribution of ribosomes along the S1mRNA relative to S4 mRNA implying that ribosome activity is the rate-limiting step of S1 mRNA translation (75).

The M1 mRNA translation efficiency is very high in vitro and very poor in vivo. The 5' noncoding region of M1 mRNA can stimulate translation of S1 mRNA and its initiating codon is "strong" (72). One explanation for the difference in M1 mRNA translation in vitro versus in vivo was that in vivo translation is initiated at the "weak" initiation codon located 49 codons downstream of the "strong" first AUG codon (76). This was not confirmed by other researchers (77) and analysis of the differences in M1 mRNA translation in vivo and in vitro awaits further investigation.

In addition to the regulation of the rate at which individual proteins are translated, translation of viral versus host-cell mRNA is regulated. Studies have shown a gradual decrease in host protein synthesis and increase in viral protein synthesis after reovirus infection. The mechanism of gradual shutoff of host protein synthesis is not known but might be partly dependent on the transition to cap-independent translation. It was shown that the proportion of uncapped mRNA increases during infection as a result of a transition from cap-dependent to cap independent translation induced by reovirus (32). Reovirus progeny subviral

particles in which capping enzymes are masked are shown to synthesize uncapped mRNA (78). At the same time σ3 is added to parental particles rendering them transcriptionally incompetent (79).

Studies of σ1 and σ3 proteins indicate the participation of viral proteins in regulation of host processes. The S1 gene products, σ1 and σ1NS, are proposed to be involved in inhibition of DNA synthesis of host cells although other viral components are required to elicit the effect (80). It is of interest that σ1NS unlike all other reoviral proteins was localized, in part, to the cell nucleus (81). The binding of σ1 protein to the murine L929 cell receptor was shown to induce programmed cell death (apoptosis). The mechanism of this process is unknown but may be linked to a receptor-associated signaling pathway that inhibits cellular DNA synthesis. The M2 gene (μ1 protein) is also involved in apoptosis induction (82). The σ3 protein was shown to interfere with interferon induced antiviral activity, to preserve the initiation of mRNA translation (83, 84) and to stimulate reporter gene expression in vitro (85, 86). On the other hand σ3 was shown to inhibit host cell protein synthesis (87).

1.3.5 Assembly

Reovirus morphogenesis occurs within the structural matrix of infected cells called "viral factories". Assembly steps were observed on the cytoskeleton with dsRNA appearing first followed by μNS and σ3 proteins and later in assembly by the rest of viral proteins. It was also proposed that μNS can serve as an anchor to the cell matrix for structures involved in viral assembly (88, 89). Studies using monoclonal antibodies confirmed previous findings identifying proteins that form complexes with ssRNA as μNS, σNS and σ3. Complexes containing dsRNA have, in addition, λ2 protein (90).

Isolated σNS and σ3 proteins were identified as ssRNA binding and dsRNA binding proteins respectively (91). The dsRNA binding motif was localized to an 85 AA domain of basic residues at the carboxyterminal fragment of σ3 (22, 92, 93). The σ3 protein also contains a zinc binding site at the amino terminus which correlates with its stability (22, 94). The zinc and dsRNA biding motifs are highly conserved (95).

Studies of ts mutants defective in replication and assembly mapped the mutations to segments S3 (σNS) and L1(λ3) for replication deficient mutants and to S2 (σ2) for assembly deficient mutants (96, 97). A region near the carboxy terminus of the σ2 protein was shown to be essential for assembly, probably due to interactions with other σ2 proteins (98). Clearly all structural and non structural proteins are involved at many different stages of the assembly process.

The assortment process proceeds concomitantly with assembly and ensures that one and only one of each of 10 genome segments is encapsidated into the progeny virion. Comparison of single stranded RNA containing complexes (ssRCC) with dsRCC revealed that assortment is coupled to replication of (+) ssRNA to dsRNA segments (90). Replicase activity has been isolated from infected cells (99). "Replicase" particles were purified and were found to have a density of 1.35 g/ml. They were found to be composed of ssRNA, partially dsRNA and dsRNA and all the virion structural proteins. Another fraction in the gradient - 1.42 g/ml - "transcriptase particles" contained dsRNA only with core structural proteins and μNS (100). A whole array of other precursors of mature particles was identified as well. Immature subviral particles were characterized by a lack of A-rich oligonucleotides, presence of μNS and differences in the core and outer shell components (101).

Reovirus infection results in the lysis of the cell and release of mature virions.

1.4 Reoviral pathogenesis studied with reovirus reassortment and anti-reovirus antibodies

1.4.1 Reovirus reassortment

Reassortment is the mechanism that allows the formation of new combinations of genome segments derived from two parental viruses on co-infection. It occurs when segments are chosen from the pool for encapsidation and results in progeny virus that contain mixed genetic information from both parents (102).

T3 reovirus easily generates deletions mutants whereas T1 does not. This could indicate a different mechanism of reassortment for these two viruses.

Analysis of the ability of T1xT3 reassortants to generate mutants on high MOI passage demonstrated that T1xT3 reassortants were able to delete not only L1 (103, 104) and L3 genome segments (as parental T3 virus) but L2 and M1 as well (37, 105). The capacity of reassortants to produce deletions was mapped to two genes : M3 and L2. Deletions of the M1 gene were observed when the M3 and L2 genes in reassortants were of heterologous origin (105).

Sequencing analysis of the genome segments of reassortants have identified mutations necessary for reassortment to occur. They are different depending on the type of acceptor virus. T3 reovirus accepting T1 or T2 genome segments has $G^{624} \rightarrow A$ and $G^{74} \rightarrow A$ mutations in its S4 genome segment. The mechanism of T1 reassortment is completely different. Each segment accepted by T1 reovirus has to have mutations that render it more similar to the T1 genome segment it replaces (106).

1.4.2 Pathogenesis of reovirus infection

Reovirus reassortment was used to map the functions of viral proteins during viral multiplication and within viral structures and has been useful for analysis of reoviral pathogenesis. Reovirus is ubiquitous in its geographic distribution and is able to infect almost all mammals. Serological data indicate that humans are repeatedly infected with reovirus over their life span (107), however, reovirus infection is not linked to any known human disease. In rodents reovirus infection causes systemic illness providing a model system to study pathogenesis. Studies in mice have demonstrated that serotypes 1 and 3 differ in their tropism for different organs. Type 1 reovirus grows well in intestinal tissue whereas type 3 does not. Similarly reovirus T1 spreads to spinal cord via the bloodstream, whereas T3 spreads via the nerves. In the CNS, T3 infects neurons, whereas T1 infects ependymal cells and the anterior lobe of the pituitary.

The S1 gene is responsible for the different reovirus tropisms within the CNS and for different patterns of spread to the CNS. The S1 and L2 genes have been demonstrated to be responsible for the different capacity of reovirus T1 and T3 to survive in the gut (108). Other studies indicate a role for the S1 gene in serotype 3-induced biliary atresia in mice (109). The M2 gene has a role in interference following mixed infections of reovirus (110). Examination of three serotypes of reovirus revealed that reovirus-induced acute myocarditis is caused by the "reoviral vertex"proteins (λ2, λ3, μ2 and σ1) (111). In a study with severe combined immunodeficient (SCID) mice the same four viral genes: S1, L2, M1 and L1 determined increase in T1 virulence and two; L1 and L2, were determinants of severity of hepatitis and viral titer in brain, intestine and liver. The M1 gene was independently important for virulence

of T1, for liver titer of reovirus and severity of hepatitis, whereas the S1 gene was critical for increased intestine and brain reovirus titers (112). The M1 gene was also shown to determine the ability of reovirus T1 to grow in cultured bovine aortic endothelial cells (113) and in heart cells (114).

The mechanism of serotype dependent titer and organ tropism depends on which gene is involved. S1 acts through its product σ1 during virus binding to the host cell and M2 (μ1, μ1N /μ1C) affects the penetration of the host cell. L1 and L2 segments (λ3, λ2 proteins) act at the level of genome transcription and replication, whereas S2 (σ2) may control the efficiency of assembly. The roles of λ1 (L3) and μ2 (M1) are unknown.

Antibody studies have been used to elucidate the role of viral proteins in reoviral multiplication and pathogenesis as well as their location within virion structures. Monoclonal antibodies against σ1, σ3 and λ2 were able to neutralize infectivity of reovirus, inhibit hemagglutination and aggregate reovirus particles (115). σ3 and μ1-specific monoclonal antibodies were shown to inhibit internalization and intracellular proteolytic uncoating (116). These antibodies were shown to protect against different serotypes of reovirus despite the different pathogenic strategies of these viruses (117).

1.5 Physical and chemical stability of reovirus

Reovirus is stable at RT and is usually stored at 4°C. It is resistant to a wide range of pH and temperatures conditions. Elevation of temperature results in a gradual loss of infectivity. Reovirus is resistant to 3% formalin, 2% lysol, 1% hydrogen peroxide, 1% phenol and ether. Brief exposure to 70% ethanol is not deleterious but longer exposure to 70 % ethanol or brief exposure to 95% ethanol is virucidal. Sodium hypochlorite (800 ppm

chlorine) and Wescodyne (1/200) are also potent virucidals. Reovirus is sensitive to UV treatment and certain dyes (neutral red, toluidine blue) (53, 108). It is also sensitive to storage in low ionic strength buffer which results in its degeneration into subunits (118).

More detailed studies revealed structural changes in virions following different physical and chemical treatments. pH 11.8 treatment results in the removal of λ2 protein (21). Other treatments: pH 11, 2.5 M guanidine-HCl, 1% SDS, 1% phenol, 33% ethanol and heating to 55°C results in the alteration of outer shell proteins σ1, σ3, and μ1/μ1C (119). Treatment of virions with 1 M sodium thiocyanate results in the removal of the outer protein shell from reovirus (120). Partial removal of the outer protein shell can be achieved with 0.5% SDS (121). The outer shell of the virion can also be removed with chymotrypsin and is routinely used in reovirus research to generate transcriptionally active particles (3, 53, 66, 67). Effects of 4-6 M urea and 50% formamide have also been assessed and were shown to result in partial degradation of reovirus particles (2, 3).

1.6 Progress in the development of rescue systems for the introduction of cDNA genes into viruses

Based on the mode of replication viruses are divided into DNA and RNA viruses with the latter further divided to (+) and (-) strand viruses. The "plus" and "minus" strand division relates to the nature of genomic RNA. Genomic RNA that serves as message on infection is referred to as "+" whereas genomic RNA which serves as a template on which message is transcribed by viral polymerases is referred to as "-". This distinction is essential in the design of viral expression systems since deproteinized RNA of (+) but not (-) strand viruses can

initiate infection cycles. To be infectious, the RNA of (-) strand viruses must be supplemented with nucleocapsid protein and viral polymerase.

The development of viruses as vectors and expression systems was first accomplished for DNA viruses and (+) strand RNA viruses (Vaccinia virus, Adenovirus, Coronavirus). Proteins produced by recombinantVaccinia T7/CITE (cap independent translation enhancer) virus are commonly used for functional/ structural studies. Vaccinia virus based vectors are currently in trials for tumor therapy and as recombinant live vaccines (122).

1.6.1 Minus strand virus expression systems

A synthetic/foreign gene was first introduced into a negative stranded virus in 1989 using influenza virus (123). The system involved synthetic RNA, purified viral nucleocapsid protein (NP) and a helper influenza virus. Rescue of synthetic RNA allowed the study of influenza cis-acting elements controlling transcription, replication and polyadenylation as well as functions of viral proteins (123, 124). Progress has been made in stable expression of foreign proteins by influenza (125, 126) and simplification of the reconstruction system (127).

Encapsidation of synthetic and/or foreign viral genes was later accomplished for other (-) stranded viruses: Respiratory Syncytial Virus (RSV) (128, 129), Measles virus (130), Sendai virus (131) and Parainfluenza virus Type 3 (132) (Paramyxoviridae). Two members of the Rhabdoviridae; Vesicular Stomatis Virus (VSV) and Rabies virus, were rescued entirely from cDNA clones (133, 134) which contained the T7 promoter at the 5' end, and autolytic HDV (Hepatitis Delta Virus) ribozyme at the 3' end, and were transcribed by T7 DNA dependent RNA polymerase expressed cytoplasmically from recombinant Vaccinia virus.

Double-stranded RNA viruses are included in the (-) strand RNA viruses class, since the dsRNA has to be transcribed to (+) sense ss mRNA in order to start the multiplication cycle. The requirements for a successful rescue system for dsRNA are thus the same as for (-) stranded ssRNA viruses.

The expression system for yeast L-A double-stranded RNA virus includes dialyzed mature or immature virus particles (viral polymerase and nucleocapsid), cell extract containing host factors and viral synthetic mRNA (135). Expression of L-A proteins and RNA from cDNA clones allowed the study of "cis" signals and packaging (136, 137), one of the classical unsolved problems of dsRNA viruses. Design of the rescue system for bacteriophage ϕ6 led to the study of encapsidation as well (138, 139). Bacteriophage ϕ6 genome organization resembles that of reovirus since it is composed of three pieces of segmented dsRNA. A bacteriophage ϕ6 in vitro assembly system was constructed by expression of proteins and RNA from cDNA (140) and proved to be useful for introduction of foreign genes (141).

1.6.2 Development of expression systems for the Reoviridae

For the Reoviridae, construction of a system for reconstitution of viable virus started with the expression of viral structural proteins using recombinant vaccinia (mammalian reovirus) or recombinant baculovirus (rotavirus, orbivirus). Expressed proteins were able to assemble into virus-like particles (142, 143, 144). For rotavirus, particles assembled in vitro were shown to replicate native or in vitro transcribed ss (+) viral mRNA into dsRNA.

Another system was developed for rotaviruses in which cores were "opened" using dialysis conditions similar to the ones used for L-A yeast virus. "Open" particles were able

to replicate exogenously delivered native or synthetic rotaviral ssRNA (145). The in vitro "transcapsidation" of rotavirus cores with exogenously supplied proteins has been accomplished (146), which could facilitate construction of synthetic infectious rotavirus.

An in vitro expression system for mammalian reovirus has not yet been developed. Conditions were worked out in which native reoviral RNA is able to be rescued into progeny virus with the aid of viral proteins translated in vitro and a helper virus of a different serotype acting in trans (147). In this system, however neither the viral genome nor viral proteins can be manipulated in order to study their function. The studies of reoviral cis-acting sequences for transcription, replication and assembly are thus currently limited to analysis of deletion or conditional mutants (37).

The in vitro expression systems developed for (-) sense viruses are now being used to examine aspects of viral structure and multiplication that could not be solved with previously available techniques. However, the ultimate goal of all viral expression systems is to develop a packaging system that would allow encapsidation of any RNA independent of size constraints. This system should be safe and effective for infection and expression and have the ability to multiply in a wide range of cell types. It is unlikely that one system would be able to fulfill a wide spectrum of specific requirements from vectors for vaccine development to ones for gene therapy. This is why work should be continued on development and improvement of all viral rescue systems.

The reovirus system offers some advantages as a potential packaging system over the ones that are in use:

1. Biosafety. Reovirus is not linked with any known disease in humans, so it will be safe for researchers and recipients.
2. Cytoplasmic multiplication of reovirus makes it easy to cultivate and purify reovirus. Also the rescue system will not have to deal with difficulties of nuclear transport and splicing.
3. Genetic stability. Homologous recombination should not affect cloned foreign genes in reovirus because it does not occur in dsRNA.
4. Wide host range. Reovirus infects nearly all mammals and a variety of cell types so that as a vector it can target numerous hosts and organs.
5. High expression. The overproduction of certain proteins will be possible in reovirus.
6. Applicability to constitutive expression. Reovirus can produce persistent infection in vitro and in vivo, which could be exploited for, long term, constitutive expression (148, 149, 150, 151).

2. RATIONALE AND OBJECTIVES

Reovirus research is hampered by the lack of an experimental system for genetic manipulation. The main genetic tools used by reovirologists to study the location and function of viral proteins in the viral particle and during the multiplication cycle have been reassortants, ts and deletion mutants. Studies with monoclonal antibodies have also been useful in vitro and in vivo and have confirmed results obtained with other systems. However all those tools have limitations. It is only possible to study mutants that are viable in nature while all the mutations that result in lethality cannot be studied.

The availability of an in vitro expression system for reovirus would overcome this problem. Numerous vital functions for reovirus multiplication and structure could be mapped and characterized. Among the most interesting are cis-acting signals for replication, transcription, assembly and encapsidation that are known to lie within the 5' and the 3' ends of the reoviral genome. The sequence of steps required for packaging could be studied and mechanisms underlying selection of one segment of each size per viral particle could be determined. The viral replicase and transcriptase could be identified with certainty as could other viral/host factors necessary for their function.

The overall goal of our lab is to develop reovirus as an expression vector that would allow genetic manipulation of the reovirus genome and proteins. Once this is accomplished, reovirus can also be used as a system for expression of foreign genes and vaccination.

Viral expression systems have been developed for the dsRNA segmented viruses ϕ6 and rotavirus. Rotavirus belongs to the same family as mammalian reovirus (Reoviridae) and except for structural differences and some steps in assembly the overall multiplication strategy

of reovirus and rotavirus is very similar. The rotavirus system holds promise as a model that is applicable to the development of a rescue system for synthetic reoviral RNA.

My laboratory has been studying the M1 gene of reovirus and thus cDNA clones of the T1 and T3 M1 gene were available. The type 1 M1 gene was sequenced and the minimal sequences required for replication and encapsidation of M1 gene were identified in our laboratory.

Specific objectives of this thesis were:

Introduce a synthetic deleted M1 gene into the reovirus particle. From the studies of reassortants it was known that deleted M1 genes exist in nature. The T1 reovirus was used as an acceptor of a synthetic deleted T3 RNA since it does not generate deletions mutants. The deleted gene could be thus identified after transfection by its size and also its origin could be confirmed by sequencing.

Examine the fate of transfected synthetic reovirus gene in reovirus infected cells. The reason for failure to introduce synthetic genes into reovirus was addressed. Efforts were directed at the analysis of interactions of transfected, synthetic, ss (+) strand RNA with the reoviral multiplication machinery. Interactions with viral proteins and assembly into subviral particles were of particular interest.

Select a lethal M1 mutant. It should be possible to isolate an M1 deletion mutant generated by high multiplicity passage of reovirus in cells that express μ2 protein (product of the M1 genome segment). These mutants would then serve as a superb selection system for viruses that accept synthetic M1 genes. This system could thus allow genetic manipulation and functional studies of the M1 gene.

Introduce a synthetic reoviral RNA into "open core" particles. The L-A virus of yeast and rotaviruses were able to replicate exogenous mRNA after low salt dialysis. This method seemed feasible for reovirus as well. Various physical and chemical treatments were tried in order to "open" reovirus particles.

Examine the transcription activity of reoviral cores and virions after various chemical and mechanical treatments. The reoviral transcriptase and replicase are not active in soluble form. Efforts were made to alter the structure of particles while maintaining transcriptase and replicase activity. The dependence of transcriptase activity on structure integrity and its alteration was studied.

3. MATERIALS AND METHODS

3.1 Bacterial culture and plasmid isolation

DH5α and DH5αF' (Gibco-BRL) carrying plasmids were grown in culture in order to produce plasmid DNA. For small scale preparations of the plasmids (mini-preps), colonies were picked with toothpick from LB (see Appendix I) agar cultures in Petri dishes containing 100 μg/ml ampicillin (Boehringer Mannheim) and transferred to 2 ml of 2 YT (see Appendix I) with 50 μg/ml ampicillin liquid culture. Culture was incubated O/N at 37°C with agitation. For larger scale preparations, an overnight culture was used to inoculate 200 ml of 2 YT or TB (see Appendix I) which was incubated O/N with shaking (152).

3.1.1 Mini preps

Two methods were used: lysis by alkali and lysis by boiling. In the alkali method (153) the O/N culture was spun down and resuspended in 200 μl of solution 1 (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8) containing 4 mg/ml of lysozyme (Sigma). After 5 min. at RT 400 μl of 0.2 M NaOH plus 1% SDS were added, mixed and incubated 5 min. at RT. 300 μl of 7.5 M NH_4OAc was then added and the content of the tube was mixed and incubated on ice for 10 min. followed by a 3 min. centrifugation at 10000 RPM at 4°C in a microcentrifuge. Isopropanol (500 μl) was added to the supernatant and sample was incubated on ice for 10 min. The DNA was pelleted for 15 min. at 10000 RPM at 4°C, washed with 70% ethanol and air dried. The pellet was resuspended in 100 μl of TE pH 8 and spun for 2 min. at 14000 RPM. Supernatant containing DNA was transferred to a fresh tube.

In the lysis by boiling method (152), the bacterial pellet was resuspended in 350 μl of STET buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 5% Triton X-100) and

25 μl of 10 mg/ml lysozyme in 10 mM Tris-HCl pH 8 was added. The mixture was boiled for 40 sec. and centrifuged at 14000 RPM for 10 min. at RT. The pellet was removed from the microcentrifuge tube with a sterile toothpick. The DNA was precipitated with 40 μl of 3 M NaOAc pH 5.2 and 420 μl of isopropanol and incubated 5 min. at RT. The DNA was pelleted at 14000 RPM for 5 min. at 4°C, washed with 70% ethanol and dissolved in TE (see Appendix I) pH 8. The DNA prepared by those two methods was stored at -20°C and used mainly for restriction digestion. If further purification was required, the DNA was extracted with phenol/chloroform (chloroform : isoamyl alcohol 24:1), followed by chloroform extraction, RNase A digestion (20 μg/ml, 20 min., 37°C), another phenol/chloroform extraction and purification on an Sephacryl S-400 spun column. Sephacryl S-400 (high resolution) (Pharmacia) suspended in TE pH 8 was packed in a 1 ml syringe and washed 2 times with 100 µl TE before the sample was applied. DNA purified on S-400 was precipitated using 1/2 volume of 7.5 M NH_4OAc pH 7.5 and 2.5 volume of 100% ethanol.

3.1.2 Midi preps

The DNA was isolated from 200 ml of bacterial culture using Qiagen columns and kit reagents, following the manufacturer's instructions. The extracted DNA was used for restriction digestion, cloning and transfection experiments.

3.2 Methods used in plasmid constructions

3.2.1 PCR

The polymerase chain reaction (PCR) was performed in a final volume of 100 μl , that included the following : 1 ng of DNA or reverse transcription product, 100 ng of each primer, 0.2 mM of each dNTP (Pharmacia) 2.5 U of Taq DNA polymerase, 0.1% NP-40, 50 mM

KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$ and 0.01% (w/v) gelatin (37). An additional 2 mM $MgCl_2$ was sometimes included in the reaction. All the PCR reactions were performed in a Hybaid Thermal Reactor (Bio/Can Scientific Inc), using usually 30 cycles, consisting of 94°C denaturation for 1 min, 72°C amplification for 2 min, and 48°C annealing for 1 min. A 5 μl portion of the 100 μl PCR reaction was analyzed on a 1% agarose gel before the remainder was purified by extraction with phenol/chloroform, followed by chloroform extraction, Sephacryl S-400 spun column purification and ethanol precipitation.

In preparation for ligation into plasmids the ends of PCR products were repaired and phosphorylated. The DNA pellet was dissolved in 12.3 μl of H_2O and supplemented with stock buffers to result in 50 mM Tris HCl pH 7.6, 10 mM $MgCl_2$, 0.08 mM of each of the 4 dNTP's, and 1.15 U of the Klenow fragment of DNA polymerase I. The reaction was incubated at RT for 40 min. and then at 70°C for 5 min. Then the reaction was supplemented to contain 5% polyethylene glycol 8000, 1 mM ATP, 1 mM DTT and 0.2 mg BSA. The 5' ends of PCR products were then phosphorylated by the addition of 2.5 U of T4 polynucleotide kinase for 30 min. at 37°C. The reaction was stopped by heating at 70°C for 5 min, and then the PCR products were ligated to dephosphorylated pGEM-7Zf(+) which was digested with Sma I, using 2 U of T4 ligase, O/N at RT.

3.2.2 Bacterial transformation

"Library Efficiency DH5αF'™" and" MAX Efficiency DH5α™" competent cells for chemical transformation were purchased from GIBCO BRL and the transformation was performed according to the manufacturer's instructions with some changes. Instead of 100 μl aliquots, 50 μl of competent cells were aliquoted into 15 ml propylene tubes on ice. The

ligation mixtures were diluted 5-fold and 1 μl of the diluted mixture was added to competent cells. The competent cells were incubated with DNA on ice for 30 min. Heat shock was performed by transferring the tube to a 42°C water bath for 45 seconds, followed by chilling on ice for 2 min. The cells were allowed to express the ampicillin gene by addition of 300 μl of SOC (see Appendix I) medium and incubation for 1 h at 37°C with vigorous shaking. Aliquots (10-100 μl) were plated on LB (see Appendix I) plates containing 100 μg/ml ampicillin.

To prepare bacterial cells for electrotransformation, 1 liter of 2 YT (see Appendix I) was inoculated with 10 ml of a fresh overnight culture of DH5α. The cells were grown at 37°C with vigorous shaking, until the OD_{600} was between 0.8 and 1. The culture was chilled on ice for 15-30 min. and centrifuged at 4000xg for 15 min. The supernatant was removed, the pellet resuspended in 1 liter of cold water and spun again at 4000xg for 15 min. This washing process was repeated with 0.5 l of water and 20 ml of 10% glycerol. Cells were finally resuspended in 3 ml of cold 10% glycerol, frozen on dry ice and stored at -70°C.

Electroporation was performed using a Cell-Porator (Gibco-BRL) apparatus essentially as described by the manufacturer. Electrocompetent cells (20 μl) were mixed with 1 μl of 4-fold diluted ligation mixture put into the electroporator chamber, and pulsed at 2000 Volts. The electro-transformed cells were removed from the chamber with a Pasteur pipette, diluted to 2 ml with SOC and incubated for 1 h at 37°C with shaking followed by plating on LB plates containing 100 µg/ml ampicillin.

The α complementation test was used to screen bacterial colonies for the presence of recombinant plasmid after ligation. The α complementation test relies on the ability of

plasmids to express and complement the β-galactosidase function in bacterial cells. The function of this enzyme is manifested by blue colonies in the presence of X-gal. However if the coding region of β-galactosidase gene in the plasmid is interrupted by insertion of foreign DNA, then the blue product is not made and the bacteria carrying recombinant plasmids form white colonies in the presence of X-gal (154). Ten μl or 100 μl of transfected bacteria grown in SOC were mixed with 100 μl of 0.1 M IPTG and 20 μl of 80 μg/ml Bluo Gal (in dimethylformamide) and plated on LB agar.

3.2.3 Colony hybridization

To shorten the process of identification of plasmid constructs that contain inserts, colony hybridization was performed (155). Nitrocellulose membranes (Gene Screen DuPont) were placed on LB/100 μg/ml ampicillin plates and the colonies were grown O/N. Alternatively colonies were transferred to the nitrocellulose membrane by putting the membrane onto the colonies on the LB plate for 2 min. and peeling off the filter. Nitrocellulose filters were then transferred, colony side up, to Whatman 3MM paper and soaked with 10% SDS for 3 min. Next, they were transferred for 5 min. to 3MM paper that was soaked with denaturing solution (0.5 M NaOH, 1.5 M NaCl). Following the denaturation, filters were placed for 5 min. on 3MM paper soaked with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8), and then transferred for 5 min. to 3MM paper soaked with 2x SSC (see Appendix I). Filters were rinsed in 2x SSC for 5 min. with shaking and dried at room temperature. The dry filters were baked for 2 h at 80°C.

Primers that were complementary to the insert sequence were used for hybridization with the DNA transferred onto nitrocellulose filters. One hundred ng of primer was labeled

with 50 μCi of [γ^{32}P] ATP and 5 U of polynucleotide kinase in the presence of 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA. The reaction was incubated for 30 min. at 37°C and stopped at 70°C for 5 min. The primers were purified from unincorporated nucleotides by Sephadex G-50 (Pharmacia) spun column centrifugation. The nitrocellulose filters were prehybridized for 2-3 h at 65°C in 5x SSC, 50x Denhardt's (see Appendix I) and 1% SDS. Then the ^{32}P labeled primer was added and the filters were hybridized O/N at 42°C. Filters were washed 4 times in 1x SSC, 0.1% SDS for 20 min. at RT, followed by 2 washings with 1x SSC, 0.1% SDS at 42°C. Filters were placed on 3MM Whatman paper soaked with 2x SSC, covered in Saran Wrap and exposed to x-ray film.

3.2.4 Restriction digestion

Most of the restriction enzymes were purchased from Pharmacia. One-Phor-All buffer Plus was used in the restriction reaction at the concentration indicated by the manufacturer to suit each specific restriction enzyme. Each restriction enzyme was diluted at least 10 fold depending on the activity of the enzyme and length of incubation time. Bsm I restriction enzyme was purchased from New England Biolabs and was used with the NEBuffer 2 supplied by the manufacturer. The reaction was incubated at 65°C O/N under paraffin oil.

3.2.5 Agarose gel electrophoresis

Agarose was prepared with 1x TBE (see Appendix I) at a concentration of 0.8-1.2% and gel electrophoresis was carried out in 1x TBE buffer. DNA was loaded in 0.05% bromophenol blue, 0.05% xylene cyanol FF, 5% glycerol and 10 mM EDTA and electrophoresed at 5 V/cm. The DNA separated on the gel was stained with 0.5 μg /ml

ethidium bromide for 30 min. Pictures of agarose gels were taken using the Gel Print 2000I (Bio Photonics Corporation) digital camera and printed by a video copy processor (Bio/Can Scientific).

3.2.6 Electroelution

Electroelution was performed in glass tubes (156). The bottom of the tube was sealed with 0.8% agarose, before a dialysis bag with 500 μl of 0.5x TBE (see Appendix I) was attached and tubes were placed vertically in the electroelution apparatus. DNA bands were identified on the agarose gel using a long wavelength ultraviolet lamp. Pieces of agarose containing DNA were put inside the glass tube and the tube was filled with 0.5x TBE. Electroelution was performed in 0.5x TBE at 100 V O/N. The DNA which migrated into the dialysis bag was extracted 3 times with n-butanol. Then it was extracted with phenol/chloroform three times and purified on a Sephacryl S-400 spun column. The purified DNA was then precipitated with 0.5 vol. of 7.5 M NH_4OAc pH 7.5 and 2.5 vol. of 100% ethanol.

3.3 In vitro transcription of plasmid DNA

In order to prepare the DNA for run off transcription, 20 μg of the plasmid DNA was cut with the appropriate restriction enzymes and analyzed on an agarose gel to assure that the digestion was complete. Before the transcription reaction was started the DNA was treated with proteinase K to inactivate any RNase that may be present in the DNA. This reaction mixture contained : 0.5% SDS, 50 mM NaCl, 10 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM EDTA pH 8 and 100 μg/ml of Proteinase K (Boehringer Mannheim). The reaction was incubated for 30 min. at 37°C. The DNA was then extracted with phenol/chloroform,

followed by chloroform extraction and ethanol precipitation. Following the proteinase K treatment 3' overhangs created by restriction enzyme digestion were removed by treatment with T4 DNA polymerase (Pharmacia). In this reaction the DNA was resuspended in 20 μl of One-Phor-All Buffer PLUS, containing 0.08 mM of each dNTPs and 2U of T4 DNA polymerase. The reaction was incubated for 15 min. at 12°C and stopped by heating at 70°C for 10 min. DNA was extracted with phenol/chloroform and once with chloroform. It was purified on a Sephacryl S-400 spun column and ethanol precipitated.

Five μg of DNA prepared in this way was used for transcription (157). DNA was incubated with 20 mM Tris-HCl pH 7.5, 30 mM $MgCl_2$, 1 mM spermidine, 5 mM NaCl, 10 mM DTT, 100 U of RNAsin (Pharmacia) and 67 U of T7 RNA polymerase in a 100 μl reaction. One to 0.5 mM of each of the 4 NTPs was added if there was no cap analog and no label included in the reaction. In some reactions the cap analog : $m^7G(5')ppp(5')G$ or $m^7G(5')ppp(5')Gm$ (sodium salt) (Pharmacia) was included at a concentration of 0.5 mM, and the GTP concentration was reduced to 0.05 mM. If [α^{32}P] UTP was included in the reaction the "cold" UTP concentration was reduced to 0.2 or 0.1 mM. In some transcription reactions 1 mM SAM (S-adenosylmethionine) was included. The reaction was incubated for 1 h at 37°C. Sometimes another 67 U of T7 RNA polymerase and 36 U of RNAsin was added and the reaction was continued for another 1 h. After RNA synthesis, DNA was removed from the reaction mixture with 7.5 U of DNase I for 15 min. at 37°C. Then the RNA was extracted with phenol/chloroform and precipitated with 0.5 vol. of 7.5 M NH_4OAc pH 7.5 and 2.5 vol. of 100% ethanol. The DNase I step was omitted for the ^{32}P labeled transcripts which were

used for L929 cell culture transfections. All ^{32}P labeled transcripts were additionally purified on Sephadex G-50 columns in order to remove unincorporated nucleotides.

3.4 Preparation of dsRNA from (-) and (+) sense ssRNA

One hundred μg of (-) and (+) sense ssRNA were each dissolved in 30 μl of annealing buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide), mixed and incubated at 65°C for 2 min., followed by O/N at RT. Double stranded RNA was extracted with phenol/chloroform and ethanol precipitated. dsRNA prepared in this way was used for transfections.

3.5 Transfection

The ssRNA transcripts alone or together with synthetic ds RNA (^{32}P labeled or not) were transfected into reovirus infected cells at different times after infection. Synthetic cationic lipids, Lipofectin and Lipofectamine (158) were used for transfection following the manufacturer's protocol (Gibco-BRL). The cells to be transfected were freshly split to be 50-90% confluent. They were washed once with PBS (see Appendix I) and once with MEM without serum or antibiotics prior to transfection. This MEM was used throughout transfection experiments. The RNA was dissolved in 100 μl of MEM and combined with 50 μl of Lipofectin or 15-30 μl of Lipofectamine supplemented to 100 μl with MEM in a 15 ml tube, mixed gently and incubated at RT for 10 min. Then 1.8 ml of MEM was added to the mixture and the content of the tube was transferred onto cell cultures. In experiments aiming to introduce a synthetic gene into reovirus, the transfection mixture was incubated with infected cells for 5 h and then replaced by MEM containing 5% FBS. In the experiment where the fate of transfected RNA was examined, the medium was not changed. In those

experiments the cells were washed twice with PBS (see Appendix I) or other specific buffers only before harvesting.

3.6 Preparation of the cytoplasmic extracts from eukaryotic cells

3.6.1 NP-40 based method

Cells growing in 60 mm dishes were washed twice with NP-40 buffer pH 7.4 (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris pH 7.4) before the addition of 1-2 ml of NP-40 buffer, containing 0.5% NP-40 to each dish on ice (37). Cells were scraped off with a rubber policemen or bent yellow tips. The buffer in which transfected cells were harvested sometimes contained 1 mM PMSF (in isopropanol) and 20 mM vanadyl-ribonucleoside complexes. The scraped cells were transferred to a centrifuge tube, vortexed and set on ice for 5-30 min. Then the lysate was centrifuged either in a microcentrifuge for 3 min. at 12000 RPM (if the cytoplasmic extracts were used for immunoprecipitation) or 10 min. at 1000 RPM (if the lysate was later processed for ds reoviral RNA).

3.6.2 Triton X-100 based method

An alternative to the NP-40 based method was also used to extract the cytoplasmic fraction. In this method (90) Triton X-100 and DOC were used. The cells were washed twice with PBS (see Appendix I) and harvested by scraping in 0.5 ml of TMN buffer (25 mM Tris-HCl pH 7.6, 3 mM $MgCl_2$, 100 mM NaCl) which included 1 mM PMSF and 20 mM vanadyl-ribonucleoside complexes. In a microcentrifuge tube, 50 μl of 10% Triton X-100 was added, the content of the tube was vortexed, then 25 μl of 10% DOC was added. The sample was vortexed again, incubated on ice for 3 min. and centrifuged for 3 min. at 12000 RPM. The

supernatant was transferred to a new tube, the pellet was washed with 250 μl of TMN buffer containing 1% Triton X-100 and 0.5% DOC, which was pooled together with the first supernatant. The cytoplasmic extracts from reovirus infected cells prepared in this way were then either put on a sucrose gradient or immunoprecipitated.

3.7 Reverse transcription of reovirus dsRNA from cytoplasmic extracts

Cytoplasmic fractions that had been extracted with NP-40 were supplemented with 5 M NaCl to 0.45 M followed the addition of 3 volumes of 100% ethanol. dsRNA was pelleted for 15 min. at 2000 RPM, washed with 70% ethanol and dried. The pellet was dissolved in STE buffer (10 mM Tris-HCl pH 8, 0.1 M NaCl, 1 mM EDTA pH 8). One percent SDS and 200 μg/ml Proteinase K were added and samples were incubated at 37°C for 30 min. (37). Proteins were extracted twice with phenol/chloroform (chloroform : isoamyl 24:1), once with chloroform and dsRNA was precipitated with 0.1 vol of 2.5 M NaOAc pH 5.2 and 2.5 volume of 100% ethanol. The purified dsRNA was pelleted at 2000 RPM for 15 min. It was either separated on a protein gel or used for reverse transcription.

All the dsRNA harvested from a single 60 mm dish was used for reverse transcription. It was dissolved in 4.65 µl of 90% DMSO (1 mM Tris-HCl pH 7.4) and incubated for 45 min. at 50°C. Then 1 mM of each of the 4 dNTPs, 100 μg of each of two primers, 34 U of Avian myeloblastosis virus reverse transcriptase, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 6 mM $MgCl_2$, 10 mM DTT were added to a final volume of 60 µl, and samples were incubated at 42°C for 1 h (modification of 6, 159). RNA-cDNA hybrids (0.5 µl) from the reverse transcription were used for PCR.

3.8 Radioimmunoprecipitation assay

Cells were labeled with ^{35}S Met for 1-4 h prior to harvesting (160). The monolayers were washed twice with PBS (see Appendix I) and 80 μCi of ^{35}S Met (Expre ^{35}S ^{35}S[^{35}S] Protein labeling Mix) (DuPont) was added in 1 ml of prewarmed DMEM that was methionine and cysteine free. The cytoplasmic extracts were harvested using NP-40 buffer (see 3.6.1), Triton X-100 / DOC (see 3.6.2) buffer or RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 10% glycerol). One mM PMSF and 20 mM vanadyl-ribonucleoside complexes were added to all buffers before harvesting.

For immunoprecipitation, 1 ml of cell lysate or a fraction from a sucrose gradient was incubated with 300 μl of 10% protein A sepharose or 150 μl of 10% protein A sepharose 4 Fast Flow (Pharmacia) (in RIPA lysis buffer or TMN / 0.1% Triton X-100) and 1.5-5 μg of the appropriate polyclonal antibody at 4°C for 2 h on a rocking platform. In the experiments where monoclonal antibodies were used (anti-σ3 + μ1C + μ1, anti-μNS and anti-σNS, gift from dr. Joklik), 2 μg of appropriate monoclonal antibody was added to the cell lysate, incubated for 1 h 30 min. and then 150 μl of 10% protein A sepharose (TMN with 0.1% Triton X-100) were added and the radioimmunoprecipitation was incubated for another 1 h. The sepharose beads were washed twice for 20-30 min. with 1 ml of TMN with 0.1% Triton X-100 or RIPA washing buffer (100 mM Tris-HCl pH 8, 500 mM LiCl, 1% 2 ME) followed by TE (see Appendix I) washing. The beads were pelleted in the microcentrifuge for 20 sec.

In the experiments where the unbound fraction was examined for RNA content, the supernatant after immunoprecipitation was precipitated with 1/10 vol of 5 M NaCl and 3 vol of 100% ethanol, treated with proteinase K in STE buffer (see 3.7), extracted twice with

phenol/chloroform and once with chloroform. Single stranded RNA was precipitated with ethanol and analyzed in 4.5% polyacrylamide gel containing 8 M urea. To analyze the proteins, bead pellets were resuspended in protein sample buffer and separated in 7.5% polyacrylamide gel. In order to analyze protein-RNA complexes the beads were resuspended in TEN buffer (25 mM Tris-HCl pH 7.6, 5 mM EDTA, 100 mM NaCl) with 1% SDS, extracted twice with phenol/chloroform, once with chloroform, precipitated and separated in 4.5% polyacrylamide gel containing 8 M urea.

3.9 Sucrose gradient centrifugation

Sucrose gradient centrifugation was used to study synthetic RNA association with viral proteins, protein complexes and reoviral particles in cell lysates. Sucrose solutions of 4% and 40% were made in TM (10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$) with 0.15 M KCl. Sucrose solutions of 15% and 40 % were prepared in TE (see Appendix I). The continuous gradients were made using a gradient maker. The 4-40% gradients were centrifuged in a Beckman SW50.1 for 28 h at 45000 RPM or in the Sorvall TH641 rotor for 24 h at 38000 RPM at 4°C (91). The 15-40% gradients were centrifuged in the TH641 rotor for 2.5 h or 5 h at 38000 RPM at 4°C. The fractions were taken from the bottom by collecting drops from the tubes punctured with a $^5/_8$ inch 25 g needle. Eight-9 fractions were collected from 12 ml centrifugation tubes and 5 fractions were collected from 5 ml tubes.

The fractionated cell lysates underwent radioimmunoprecipitation, scintillation counting or were treated with 200 μg/ml Proteinase K in 1% SDS for 30 min. at 37°C, followed by two phenol/chloroform extractions, one chloroform extraction, ethanol precipitation and analysis by polyacrylamide gel electrophoresis.

3.10 Metabolic labeling of reovirus RNA in infected cells

Cells infected at an MOI of 50 were incubated for 2.5 h, then the medium was removed and 4 ml of MEM/5% FBS with 100 μCi of ^{32}P orthophosphate (Phosphorus-32, carrier-free, Amersham) and 1 μg of Actinomycin D were added (161). The infected cell monolayers were harvested by one of the methods described (see extraction of cytoplasmic fraction (3.6) and RIPA (3.8)) and were analyzed by radioimmunoprecipitation and sucrose gradient centrifugation.

3.11 Northern blot hybridization

The RNA to be probed with a ^{32}P- labeled primer was electrophoresed on a polyacrylamide gel. The gel was soaked in 0.1 M NaOH for 20 min. and then it was transferred to 2x TBE (see Appendix I) for 20 min. The last step was repeated once. Pall Biodyne B nylon membrane (Gibco-BRL) and 3MM Whatman paper cut to the size of the gel were soaked in 0.5x TBE. The sandwich made from gel, nylon membrane and sheets of 3MM paper was placed into Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The RNA was transferred onto the nylon membrane O/N at 20 V in 0.5x TBE. The nylon membrane was air-dried and baked for 1 h at 80°C. The primers were 5' end labeled with [γ^{32}P] ATP (see colony hybridization 3.2.3). The nylon membrane was prehybridized in 5x SSC (see Appendix I), 50x Denhart's and 1% SDS for 2-16 h at 65°C. Then, the ^{32}P labeled primer was added, and incubated for 2-16 h at 42°C. The blot was washed 4 times in 2x SSC plus 0.1% SDS at room temperature for 5 min. and twice in 2x SSC, 0.1% SDS at 42°C for 15 min. The nylon membrane was placed on 2x SSC soaked 3 MM Whatman paper, wrapped with Saran Wrap and exposed to x-ray film (37).

3.12 Viruses and cells

The following reovirus strains were used: serotype 1 Lang strain (T1), serotype 3 Dearing strain (T3) and the G2 reassortant (105). The G2 reovirus is a T1 x T3 reassortant, with S1 and L2 genome segments from T3 and with the rest from T1. Due to the presence of L2 from T3 and M3 from T1 this virus is able to generate deletions of the M1 gene on serial passage at high multiplicity of infection.

Reovirus was cultivated in mouse fibroblast L929 cells and normally used after 2 passages (P2). To generate M1 deletion mutants, μ2 expressing L929 mouse cells (clone T1-11-1), μ2 expressing C127 I mouse mammary tumor cells (clone T1-12) and μ2 expressing 3T3 Swiss mouse embryo line (clone T3-15-33) were used (162). These tissue culture cell lines were grown in monolayers at 37°C with 3.5% CO_2 in MEM supplemented with 5% FBS. L929 cells were also grown in suspension culture in Joklik-modified MEM supplemented with 5-10% Neonate Bovine Serum+FBS. Cells were counted every day and maintained at $1x10^6$ cell/ml.

3.13 Growth and plaque assay of reovirus

Cell monolayers were grown in T-25 flasks and were infected with reovirus. The medium was changed every 5-7 days of infection. Progression of CPE was observed every day and the virus was harvested when the cells were dead (3-20 days depending on the virus and its titer). The virus was harvested from infected cells by 3 rounds of freeze/thaw and stored at 4°C.

In order to determine the titer of harvested virus, preparations were serially diluted in PBS (see Appendix I) with 0.2% gelatin. The diluted virus (100-200 μl) was applied onto L929 monolayers in 6 well dishes and infected monolayers were incubated for 1 h at 37°C. Then 2.5 ml of overlaying medium (at 37°C) was added. The overlay contained 1% agar, 1 x 199 medium, 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.18% $NaHCO_3$. Three days later a second overlay of the same volume and ingredients was added. On the 6th or 9th day (in some experiments more than 2 overlays were applied and the time of plaque assay was extended) 2 ml of overlay containing 0.15% neutral red was added. The plaques were counted and harvested the next day. The plaques were picked using a sterile Pasteur pipette and transferred to 600 μl of PBS with 0.2% gelatin, followed by storage at -20°C.

3.14 Infection of suspension culture of L929 cells with reovirus and reovirus extraction

T1 reovirus was grown and purified by a modification of the method of Smith et al. (3). L929 cells in suspension culture were pelleted by centrifugation at 1000 RPM for 5 min. (no brake) and washed with 400 ml of Joklik MEM. Cells were infected at an MOI of 4 with T1P2 by incubation with virus for 1 h. Then the cells were diluted with Joklik MEM supplemented with 2.5% FBS to 10^6 cells /ml and incubated at 34°C for 3 days.

The virus was purified from infected cells by freon extraction. Infected cells were pelleted at 2500 RPM for 10 min. at 4°C (no brake). Pellets were stored frozen at -70°C or resuspended in 80 ml of HO buffer (10 mM Tris-HCl pH 7.4, 250 mM NaCl, 10 mM 2 ME). Then pellets were sonicated until the content of the tube was uniformly mixed. One-twentieth volume of 10% DOC and 1/2 vol of freon were added and samples were sonicated again.

Following sonication the organic and aqueous phases were separated by centrifugation at 9000 RPM for 10 min. at 4°C. The aqueous phase was transferred to a new tube and re-extracted with 1/2 vol of freon, sonicated and centrifuged. The aqueous phase was kept O/N at 4°C or applied to a 1.2 -1.4 g/ml CsCl gradient (in 10 mM Tris-HCl pH 7.4) immediately. The gradients were centrifuged in a Beckman SW28 rotor at 24000 RPM for 2 h at 4°C. After the centrifugation the virus band was collected and dialyzed in reovirus dialysis buffer (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4) O/N. After dialysis the virus was stored at 4°C in the presence of 0.01% NaN_3. The OD_{260} was measured to calculate the amount of purified virus particles; 1 OD_{260} = 184.5 μg/ml of virus, 1mg of reovirus = $1.13x10^{13}$ particles.

3.15 Preparation of cores

Cores were prepared from purified reovirus by digestion with chymotrypsin (60, 66, 21). The reaction included 1x SSC (see Appendix I), 200 μg/ml chymotrypsin and approximately 15 mg of reovirus in a 10 ml reaction. The reaction was incubated 90 min. at 37°C. To stop the reaction PMSF was added to a final concentration of 1 mM and then the core band was purified on a 1.3-1.5 g/ml CsCl gradient (the same conditions of centrifugation as for reovirus see 3.14). The core band was dialyzed O/N in reovirus dialysis buffer and the amount of cores was calculated: 1 OD_{260} = 250 μg prot/ml = $4.2x10^{12}$ core particles. Cores were stored at 4°C in the presence of 0.01% NaN_3.

3.16 Chemical and mechanical treatment of cores and virus particles

Various mechanical and chemical treatments were used in order to "open" and disassemble core and virus particles (Fig. 2). Virus particles were treated with 50%

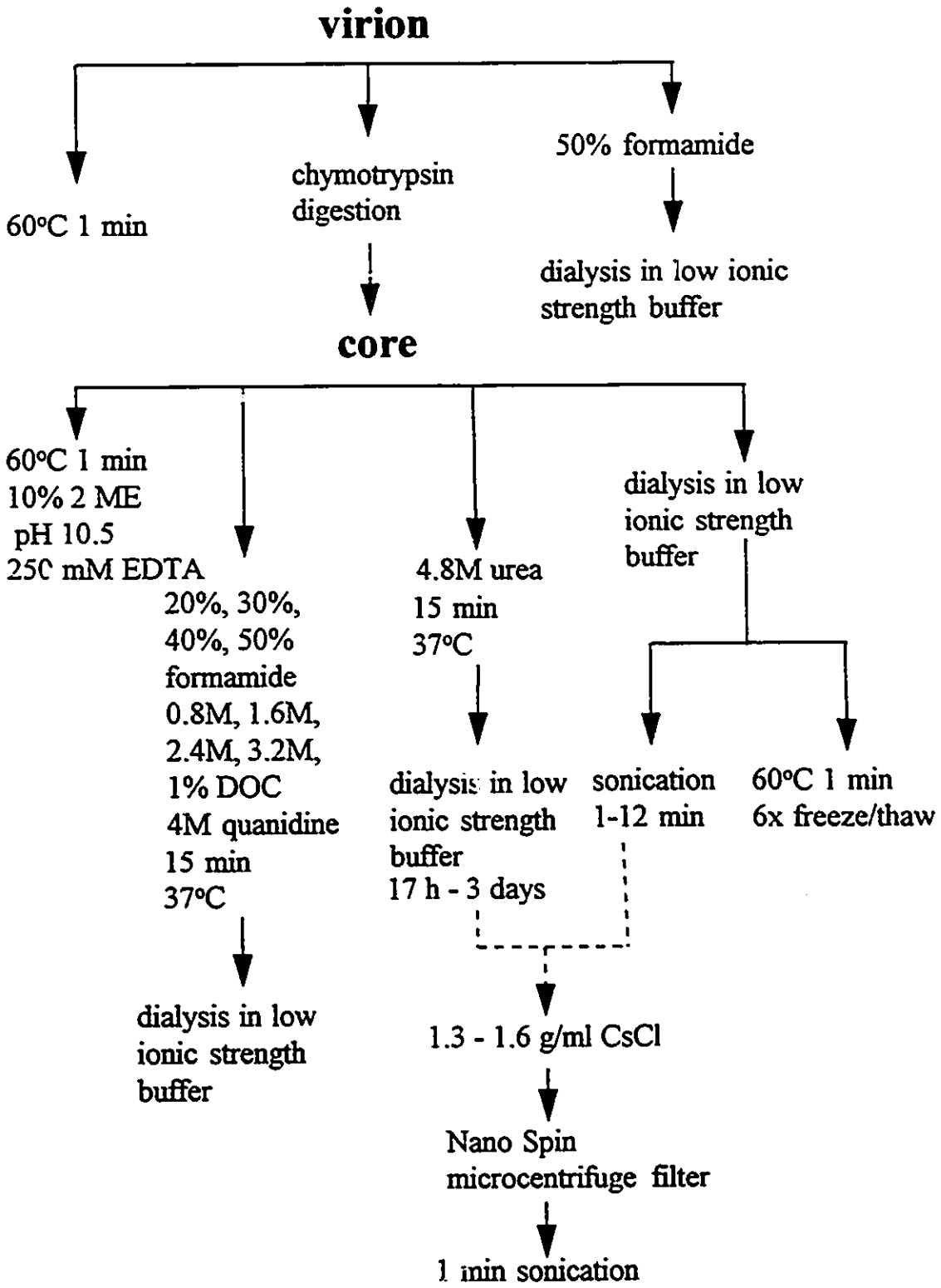
formamide. Core particles were treated with 20%, 30% and 50% formamide, with 0.8 M, 1.6 M, 2.4 M, 4.8 M and saturated urea (in the presence or not of 10 mM 2ME at RT, 40°C and 60°C), 1% DOC and 4 M guanidine. Each treatment was performed for 15 min. at 37°C (except for some urea treatments, where indicated) and was followed by dialysis in low ionic strength buffer (2 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.5 mM DTT) (145). BSA (1 μg/ml) was used during dialysis to prevent adsorption of virus particles to the dialysis membrane. Virus and core particles were also treated at 60°C for 1 min. Cores were treated with 10% 2ME, pH 10.5, and 250 mM EDTA. The cores were incubated with 10% 2ME O/N at 4°C and then they were diluted to 1% 2ME with TE pH 7.6. Dialyzed cores (dialyzed in the low ionic strength buffer) were frozen and thawed 6 times or sonicated for 1-12 min.

To assess the accessability of dsRNA following various treatments the treated particles were then digested with RNase A at a final concentration of 50 μg/ml or 100 μg/ml. Approximately 5 μg of treated virus or cores were analyzed by 7.5% polyacrylamide gel.

3.17 CsCl gradient centrifugation of 4.8 M urea treated and sonicated cores

Approximately 2 mg of treated virus (in 5% glycerol) was separated on a 1.3-1.6 g/ml CsCl (in 2 mM Tris-HCl pH 7.6, 1 mM DTT) continuous gradients in a Beckman SW50.1 rotor at 40000 RPM for 3 h. The bands collected from the gradient were desalted by centrifugation in Nanospin 30000 MWCO spun filters (Gelman). The samples were centrifuged for 5 min. at 2500xg and then rinsed with 400 μl of H_2O followed by rinsing with 400 μl of reovirus dialysis buffer, and suspension of the desalted sample in 120-200 µl of reovirus dialysis buffer.

Fig. 2. Schematic representation of physical and chemical treatments of cores and virions. The treatments were followed by RNase A digestion, transcription assay and electron microscopy.


virion
60°C 1 min
chymotrypsin digestion
50% formamide
dialysis in low ionic strength buffer
core
60°C 1 min
10% 2 ME
pH 10.5
250 mM EDTA
20%, 30%, 40%, 50% formamide
0.8M, 1.6M, 2.4M, 3.2M,
1% DOC
4M quanidine
15 min
37°C
dialysis in low ionic strength buffer
4.8M urea
15 min
37°C
dialysis in low ionic strength buffer
17 h - 3 days
dialysis in low ionic strength buffer
sonication 1-12 min
60°C 1 min
6x freeze/thaw
1.3 - 1.6 g/ml CsCl
Nano Spin microcentrifuge filter
1 min sonication

3.18 In vitro transcription/replication assay of reovirus activity

The same reaction conditions were used to examine the transcriptional activity of core preparations as for replication reactions, which usually contained synthetic ssRNA transcripts. Reaction mixtures included 100 mM Tris-HCl pH 7.2, 5 mM $MgCl_2$, 2 mM DTT, 18 U of RNAsin, 1.5% PEG 8000 and 0.8 - 5 μg of cores in a 20 μl reaction (145).

In replication reactions where ^{32}P mRNA (1 μg/20 μl reaction) was used, NTPs were used at 0.2 mM each. In the replication reaction where synthetic mRNA was not labeled and in transcription reactions, [$\alpha^{32}P$] UTP 10 μCi/20 µl reaction was added and the UTP concentration was lowered to 0.04 mM. Some replication reactions included 1 mM S-adenosylmethionine (SAM). In some replication reactions, 100 mM Tris-HCl pH 7.2 and 5 mM $MgCl_2$ were replaced by 80 mM Tris-HCl pH 8.14 and 12 mM $MgCl_2$ to optimize the conditions for the reoviral replicase.

The replication reaction was performed at 35°C, 36°C and 45°C for 4 h and the replication products were separated on 7.5% polyacrylamide gel. Occasionally ssRNA was removed from the replication reaction by RNase A digestion before gel separation. The ssRNA was digested in 1x protein sample buffer for 30 min. at 37°C with 2.5 μg/ml to 5 μg/ml of RNase A.

The transcriptase activity of different preparations of cores was examined at 35°C and 50°C (under paraffin oil). Products of reoviral transcriptase were applied to 4.5% polyacrylamide gel containing 8 M urea, in 1x protein sample buffer directly after transcription or were extracted with chloroform and phenol/chloroform, precipitated with 0.5

vol of NH_4OAc, 3 vol of 100% ethanol and 5μg of tRNA and then separated by electrophoresis on a 4.5% polyacrylamide gel.

3.19 Polyacrylamide gel electrophoresis

3.19.1 4.5% urea polyacrylamide gel for separation of ssRNA

The 4.5% polyacrylamide gel was prepared from 30% acrylamide and 0.4% N'N'-methylene-bis-acrylamide stock solution (163). The gel contained 375 mM Tris-HCl pH 8.9, 8 M urea, 0.07% ammonium persulphate (AP) (w/v), 0.6% SDS and 25 μl of TEMED (tetramethylethylenediamine) in 36 ml. The running tank buffer contained 12.5 mM Tris-HCl pH 8.3, 96 mM glycine and 0.05% SDS. The ssRNA samples contained 30% glycerol, 0.1% SDS, 10 μg/ml of bromophenol blue and 4 M urea. Protein sample buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 10 μg/ml bromophenol blue and 5% of 2 ME was used as well. The samples were heated at 70°C-100°C for 30 sec.-1 min. before loading, and separated on the gel at 120 V for 17 h.

3.19.2 Protein polyacrylamide gel

Gels containing a 3.75% stacking gel and a 10% or 7.5% separating gel were prepared according to Laemmli (163) from a stock solution of 30% acrylamide and 0.4% N'N'-methylene-bis-acrylamide. The separating gel contained 375 mM Tris-HCl pH 8.9, 0.4% SDS, 0.05% AP (w/v) and 0.05% TEMED (v/v). The stacking gel contained 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% AP (w/v) and 0.1% TEMED (v/v). The samples were loaded in 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 10 μg/ml of bromophenol blue and 5% 2 ME. Gels were run in tank buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine and

0.1% SDS. The proteins were separated in this gel system for 3-4 h at 45 mA, while dsRNA was separated for 17 h at 20 mA.

3.20 Silver staining

The silver staining protocol of Merril (164) was followed. Polyacrylamide gels were fixed in 40% methanol plus 10% acetic acid O/N. The next day they were washed twice with 10% ethanol plus 5% acetic acid for 30 min. Oxidizer (3.4 mM potassium dichromate, 3.2 mM nitric acid) was then added and incubated for exactly 10 min., followed by two washes with deionized water for 10 min. each. Silver reagent (12 mM silver nitrate) was added for 30 min. One wash with deionized water was performed for 2 min. and then the developer (280 mM sodium carbonate, 0.06% paraformaldehyde) was added. First the gel was rinsed with developer for 5 min. and then the gel was developed for 1-5 min. The reaction was stopped with 40% methanol / 10% acetic acid.

3.21 Negative staining for electron microscopy

The sample (10-20 μl) was applied on a formvar and carbon coated copper grid and incubated for 40 sec. Excess of sample was blotted off with a piece of 3MM Whatman paper. Uranyl acetate (20 μl of a 1% w/v) was then applied to the grid and incubated for 40 sec. Stain was blotted off with 3MM paper. Grids were dried O/N on 3 MM paper before analysis in a Phillips EM 300 electron microscope at an operating voltage of 80 kV.

3.22 Scintillation counting

Sucrose gradient fractions (50 μl) were analyzed in 5 ml of toluene and Triton X-100 based scintillation fluid. ^{32}P bands were cut from polyacrylamide gel and analyzed in H_2O. The model 1214 Rack beta LKB liquid scintillation counter was used.

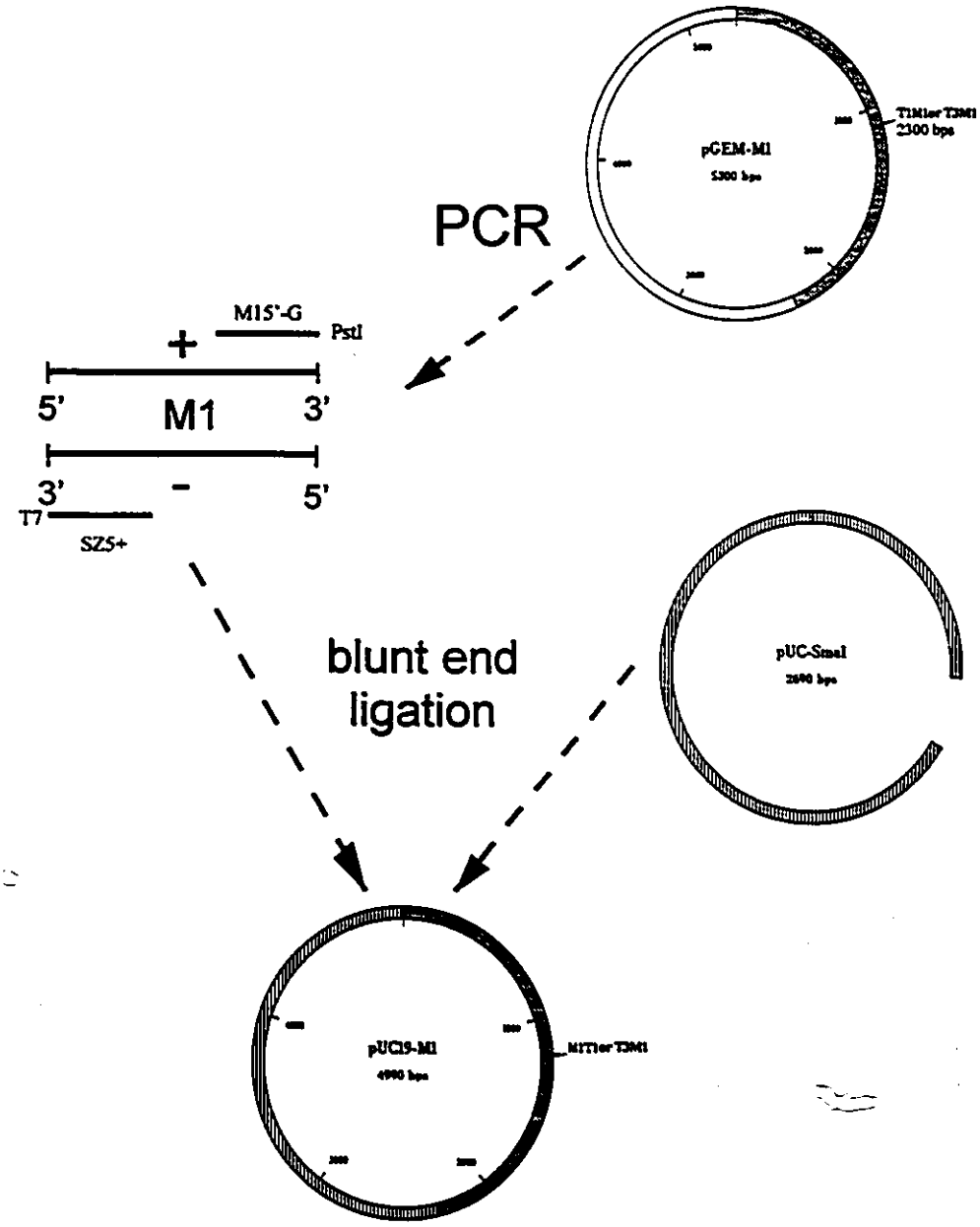
4. RESULTS

4.1 Introduction of the T7 promoter and Pst I restriction site into plasmids containing T1 and T3 M1 genome segments

In order to produce synthetic in vitro transcripts of the reovirus M1 gene, plasmids carrying this gene were modified by PCR to introduce the T7 promoter and the Pst I restriction site at the ends of the M1 gene for run-off transcription. The transcribed M1 gene was later used as a synthetic reoviral gene for transfections of reovirus infected cells. The M1 gene of T1 and T3 reoviruses had been previously cloned into pGEM-7Zf(+) (38). Two constructs containing the M1 gene, termed M1T1/pGEM-7Zf(+) and M1T3/pGEM-7Zf(+) were used for PCR.

PCR was performed using SZ5(+) and M15'(-G)(-) primers (see Appendix I) (construction shown in Fig. 3). The SZ5(+) primer contains sequence coding for the T7 promoter at the 5' end, and is complementary to the 3' end of the (-) strand of both T1 and T3 M1 genes. The M15'(-G)(-) primer contains sequence coding for a Pst I site and is complementary to the 3' end of the (+) strand of M1T1 and M1T3. The PCR amplified products were ligated to pUC19 which was cut with Sma I and dephosphorylated by CIP. The ligation reaction products were chemically transformed into Library Efficiency DH5α F'™. Colour selection was used to detect clones with inserts. Clones were further screened by restriction digestion with Pst I and Pst I/Sca I. Clones were also detected by colony hybridization using BogPriT3 (see Appendix I), which hybridizes to nt 1371-1396 of the (-) sense strand of both the T3 and T1M1 genes. Positive clones were confirmed by EcoR I, Xba I and Sal I restriction digestion. T3#20 and T1#20 were chosen for further work.

Fig. 3. Schematic representation of the cloning strategy for the T1 and T3 M1 gene for run-off transcription.

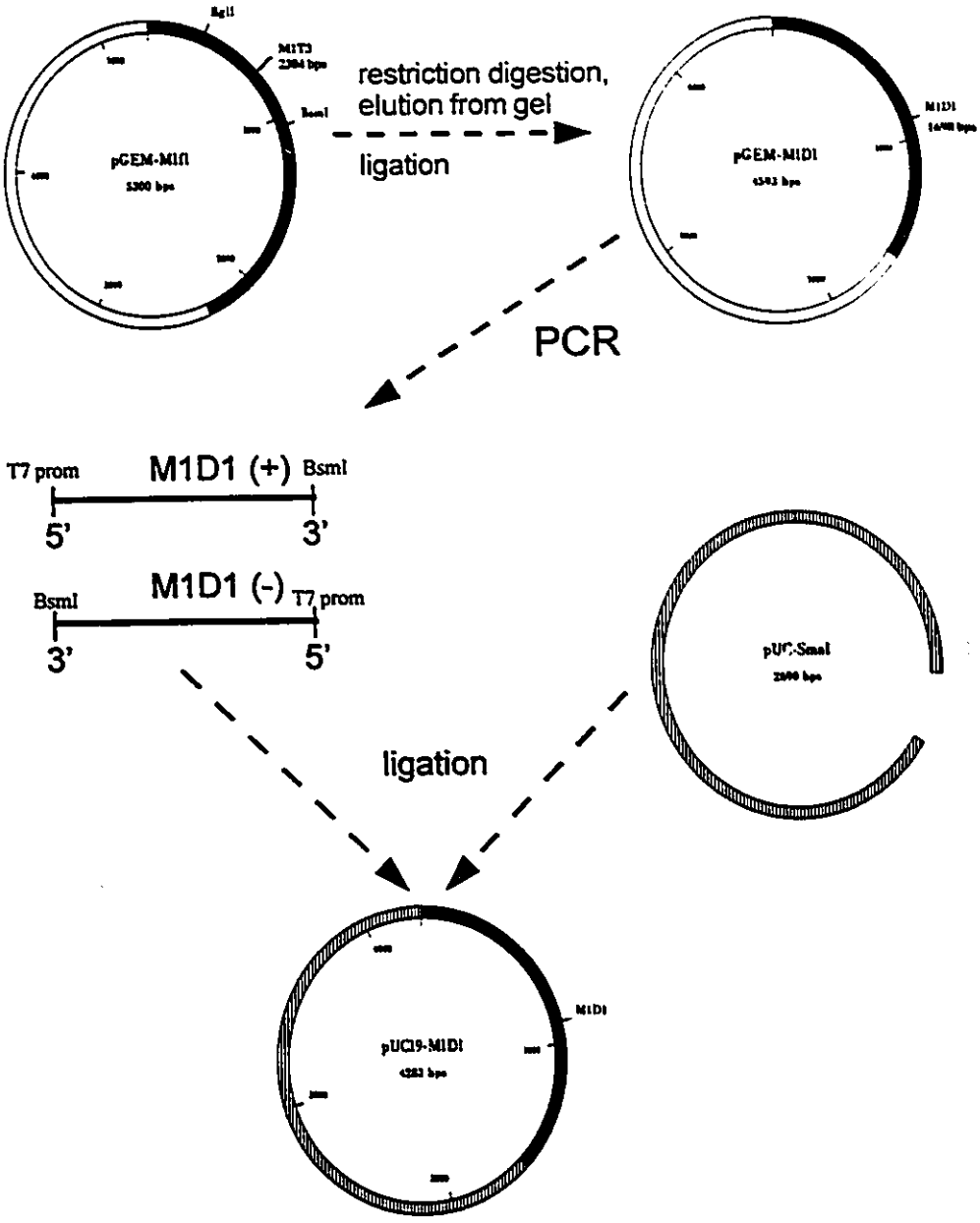

pGEM-M1
PCR
M15'-G
PstI
+
5'
M1
3'
3'
-
5'
T7
SZ5+
blunt end
ligation
pUC-SmaI
pUC19-M1

4.2 Construction of a deleted T3M1 gene (M1D1)

The deleted M1 gene was constructed in order to produce ssRNA for transfection experiments. The decreased size made it possible to distinguish the deleted M1 gene from the full size native M1 of the helper virus. The deletion was produced in the M1T3/pGEM-7Zf(+) plasmid by digestion with Bsm I and Bgl II (Fig. 4). As a result, a 706 bp fragment was deleted from the middle of the M1T3 gene. The plasmid band was purified on an agarose gel before excision and electroelution. The ends of DNA were filled in using the Klenow fragment of E.coli DNA polymerase I and ligated. The ligation mixture was electroporated into DH5α. Plasmid DNA was extracted and screened by Pst I digestion.

The T7 promoter and restriction enzyme recognition site were added to the deleted M1 gene by PCR. Prior to PCR amplification plasmid DNA was digested with Ban II and Bgl I. The primers for PCR were chosen so that two types of M1 deleted gene were obtained: one with the T7 promoter at the 5' end of the (+) strand (SZ5+, SZ7-) (see Appendix I) and another one with the T7 promoter at the 5' end of the (-) strand (SZ8-, SZ9+) (see Appendix I), both with a Bsm I site on the opposite end. PCR products were purified, the ends were repaired by Klenow treatment, phosphorylated, and ligated into pUC19 that had been digested with Sma I. Following ligation, DNA was electroporated into DH5α and plated on LB plates with colour selection. The clones were screened by restriction digestion with Bsm I and Kpn I/Xba I. Two clones were chosen for further work: #2.8, (-) sense construct, and #6.7, (+) sense construct.

Fig. 4. Schematic representation of the M1D1 construction.


restriction digestion,
elution from gel
ligation
PCR
T7 prom
M1D1 (+)
BsmI
5'
3'
BsmI
M1D1 (-)
T7 prom
3'
5'
ligation

4.3 Introduction of the synthetic deleted T3M1 gene into reovirus particle

4.3.1 Introduction of M1D1 into reovirus infected cells by transfection

Synthetic reoviral ssRNA was produced as described in 4.1 and 4.2 and was transfected into reovirus infected cells. It was expected that the synthetic ssRNA would be replicated through recognition by viral replicase, and then encapsidated into virions by T1 reovirus which was chosen as a helper virus for this study. Since T1 reovirus does not generate deletion mutants, the amplification of the M1D1 gene would indicate the successful introduction of a synthetic gene. Also, the M1D1 construct was derived from the T3M1 gene and thus it would be possible to confirm the origin of the transfected gene by its sequence.

M1D1 (+) and (-) sense constructs (see 4.2) were linearized with Bsm I so that exact 3' ends as in the native M1 (+) ssRNA would be obtained. The cut DNA was treated with T4 DNA polymerase to produce blunt-ends and used for in vitro transcription. Two types of ssRNA were made as a result: (+) sense and (-) sense. The size and quantity of ssRNA obtained by transcription was assessed by polyacrylamide gel electrophoresis. The (+) sense RNA was used for transfection of T1P2 (T1, passage 2 reovirus) infected L929 cells. The (-) and (+) sense RNA were annealed to produce dsRNA.

Two different transfection experiments were performed. In one, 50% confluent L929 cells were infected with T1P2 reovirus at an MOI of 30, one hour prior to lipofection. Lipofection was used to introduce 0.2 μg of (+) M1D1 ssRNA into infected cells. The transfection mix was removed 5 h later and the cells were incubated with MEM containing 5% FBS until cell death (4-7 days). The virus yield was then subjected to 6 serial undiluted passage.

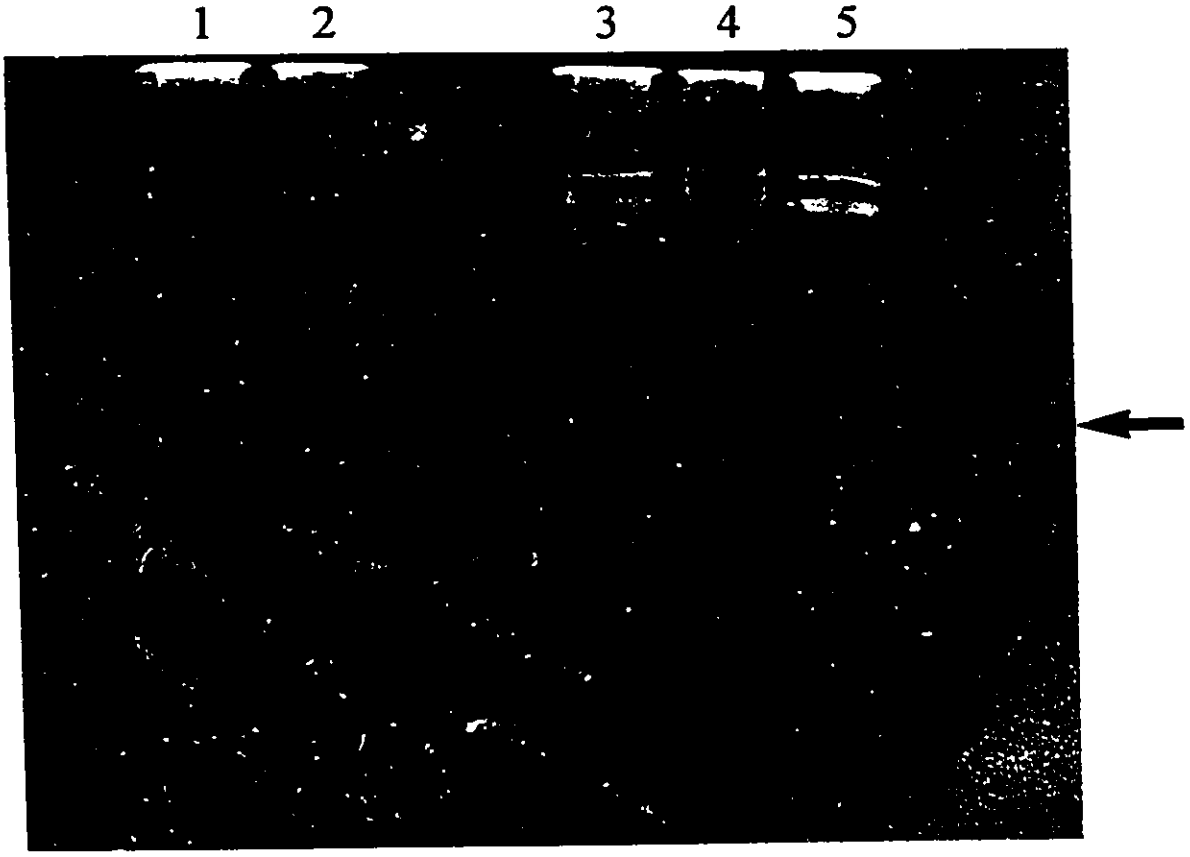
It was shown previously (147) that when native reoviral ss and dsRNA are lipofected together into L929 cells, the presence of dsRNA increases the infectiousness of ssRNA. Thus, in a second experiment, to increase the likelihood of the introduction of the synthetic gene into reovirus, ssRNA alone, as well as ssRNA plus dsRNA (0.25 μg) were transfected into 100% confluent L929 cells, infected at an MOI of 15 with reovirus. The virus was harvested and passaged at high MOI five times. During the first two passages the cell culture was additionally infected with T1P2 at an MOI of 15, 1 h prior to passage. This was done in order to increase the MOI of infection which would ensure that all cells were infected so that deletion mutants could be propagated by complementation.

4.3.2 Attempts to detect the transfected gene in progeny virus

The stocks of progeny virus were examined for the presence of the synthetic M1D1 gene after serial undiluted passage. Three methods were used to detect rescue of the deleted synthetic gene: SDS PAGE, Northern blotting and RT PCR. In each case L929 cells were infected with T1P7 from the first experiment and T1P8 from the second experiment (see 4.3.1). Three days after infection the dsRNA was extracted using the NP40 based method.

An easy method to resolve and visualize reoviral dsRNA is separation by SDS PAGE and staining with EtBr. It was expected that the deleted gene would migrate between the medium and small dsRNA reoviral segments. EtBr staining did not reveal the presence of the deleted M1T3 gene (Fig. 5).

Fig. 5. Analysis of dsRNA from reovirus infected cells transfected with the synthetic M1D1 gene by 10% SDS PAGE. (1) dsRNA from T1P2 infected cells (control), (2) dsRNA from T1 passage 8 (control), (3) dsRNA from (+) M1D1 ssRNA transfected cells, T1 passage 7, experiment 1, (4) dsRNA from (+) M1D1 ssRNA transfected cells, T1 passage 8, experiment 2, (5) dsRNA from (+) M1D1 ssRNA and dsRNA transfected cells, T1 passage 8, experiment 2. The arrow indicates the expected position of the deleted M1D1 gene on the gel.


1
2
3
4
5

Northern blotting was used as a more sensitive method to detect the transfected M1 gene. The dsRNA was transferred from a polyacrylamide gel to nylon membrane and hybridized with two ^{32}P labeled primers; M15'(+) and M15'(-) (see Appendix I), which are complementary to the 3' end of both the (+) and the (-) strands of the full length M1 and deleted M1D1 genes. Northern blot hybridization specifically detected only the full length M1 gene. The presence of transfected M1D1 gene was not detected even on overexposure (Fig. 6).

The most sensitive method for detecting M1D1 was RT-PCR. It was performed on extracts of infected cell lysates. Reverse transcription and PCR were performed using primers M15' (+) and M15' (-) (the same as for Northern blot hybridization). It was expected that both the full length M1 and deleted M1 gene (if present in the cell lysates) would be amplified. As a result of RT-PCR, only the full length M1 gene was amplified. The presence of transfected M1D1 gene was not detected (Fig. 7). It was concluded that the synthetic M1D1 gene was not replicated by reovirus.

Fig. 6. Northern blot hybridization of reoviral dsRNA to detect a transfected M1D1 gene. Double stranded RNA was hybridized with primers specific for the 3' end of (-) and (+) sense of M1 and M1D1 RNA. (1) dsRNA from (+) M1D1 ssRNA and dsRNA transfected cells, T1 passage 8, experiment 2, (2) dsRNA from (+) M1D1 ssRNA transfected cells, T1 passage 8, experiment 2, (3) dsRNA from (+) M1D1 ssRNA transfected cells, T1 passage 7, experiment 1, (4) (control) dsRNA from T1 passage 8, (5) (control) dsRNA from T1P3 . Arrow indicates the position of full length M1 gene on the gel.

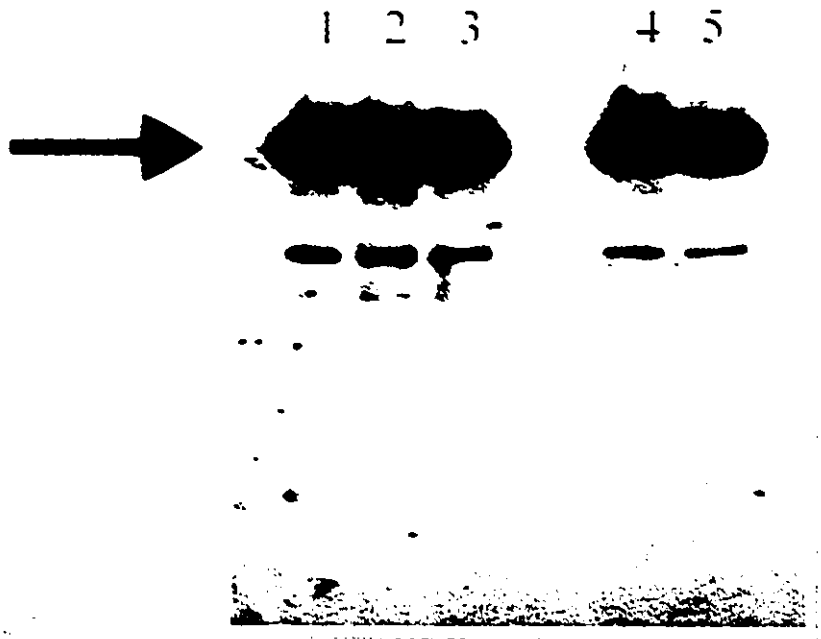
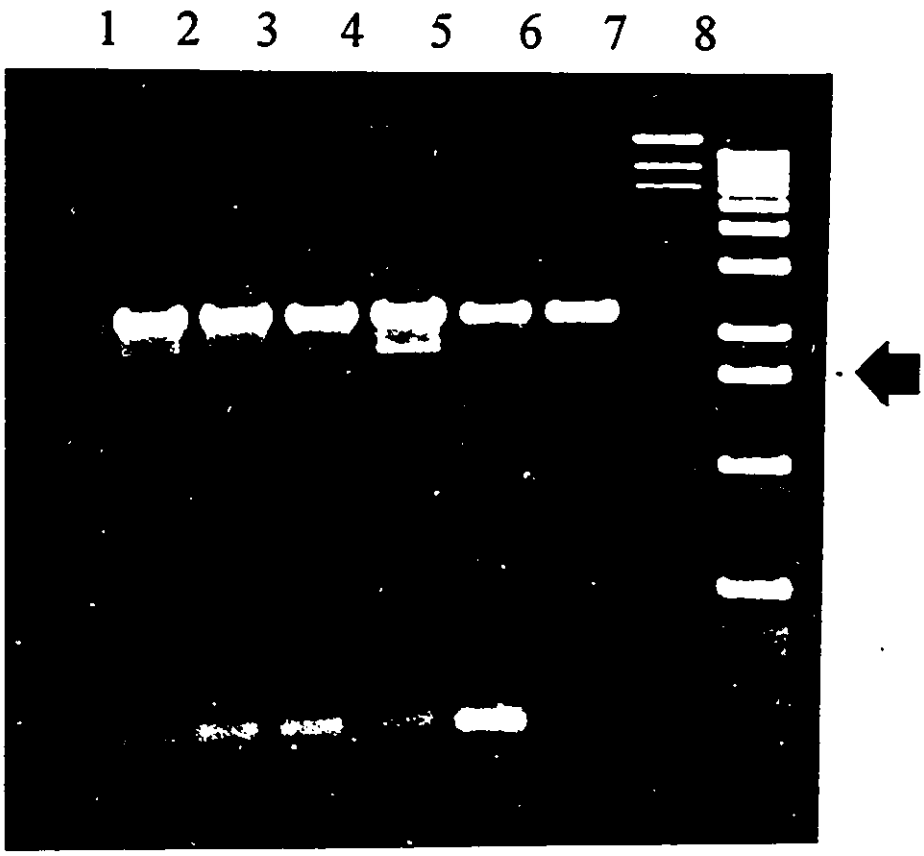

1 2 3 4 5

Fig. 7. Agarose gel analysis of RT-PCR products from cell lysates transfected with M1D1. (1) (control) lysate from T1 passage 8 cells, (2) (control) lysate from T1 passage 2 cells, (3) lysate from (+) M1D1 ssRNA transfected cells, T1 passage 7, experiment 1, (4) lysate from (+) M1D1 ssRNA transfected cells, T1 passage 8, experiment 2, (5) lysate from (+) M1D1 ssRNA and dsRNA transfected cells, T1 passage 8, experiment 2, (6) PCR performed on cDNA of M1T1 (control), (7) λ/Hind III DNA marker, (8) 1 kb DNA ladder DNA marker. The arrow indicates the expected position of the amplified M1D1 gene.


1 2 3 4 5 6 7 8

4.4 Examination of the fate of transfected M1 RNA in reovirus infected cells

Since attempts to introduce the synthetic M1D1 gene into reovirus infected cells were not successful (see 4.3.2), it was of interest to determine if synthetic ssRNA is recognized in the reovirus multiplication cycle. This study was undertaken to examine the interactions of transfected synthetic ssRNA with viral components in reovirus infected cells.

4.4.1 Association of transfected synthetic RNA with viral proteins studied by radioimmunoprecipitation

To study the fate of the transfected M1 gene in reovirus infected cells, constructs containing M1D1, as well as T1 and T3 M1 cDNA cloned into pUC19 with the T7 promoter were used to produce (+) sense ssRNA. Radiolabeled, synthetic ssRNA was transfected into reovirus infected cells. Cell lysates were examined by radioimmunoprecipitation with polyclonal reoviral antibodies for the presence of RNA/viral protein interactions. The reoviral proteins interacting with the transfected RNA and the sizes of protein complexes were of interest.

In order to prepare the (+) sense transcripts, DNA was cut with restriction enzymes for run off transcription: Bsm I was used for M1D1 and Pst I was used for T1 and T3M1 constructs. The cut DNA was treated with T4 DNA polymerase to remove the 3' overhangs (4 nucleotides after Pst I digestion and 2 nucleotides after Bsm I digestion). The purified DNA was transcribed using T7 polymerase, 1-methyl cap analog and [α^{32}P] UTP.

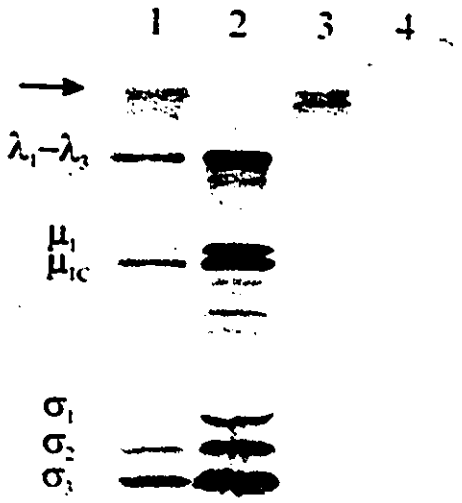
In the first experiments ^{32}P M1D1 transcript (app 0.3 μg) was lipofected into T3P2 infected (MOI of 6) L929 cells, 18 h after infection. Transfection was allowed to proceed for

1-3 h before the cell culture was harvested. The NP-40 detergent-based method was used to extract the cytoplasmic fraction from infected cells. Preliminary experiments were undertaken to determine efficiency of transfection and the stability of the transcript in infected L929 cells. Electroporation and two different cationic lipids (Lipofectin and Lipofectamine) were used to find out which method works the best for introduction of RNA into infected L929 cells. Control experiments were performed, in which synthetic ssRNA was incubated with reovirus infected cells in parallel with transfected ssRNA in order to establish that the free RNA was completely washed away during initial steps in the experiment. The preliminary experiments established that:

- lipofection is more efficient for RNA transfection than electroporation
- Lipofectamine is more efficient than Lipofectin for RNA transfection
- the RNA detected in the experiment represents only the transfected RNA since RNA added exogenously to cells was not detected (Fig. 8, lane 4)

In the following experiments proteins were labeled with ^{35}S Met before lipofection in order to study the proteins that interact with transfected synthetic ssRNA. Uninfected L929 cells transfected with synthetic RNA were used as controls in these experiments. The extracted cytoplasmic fraction was immunoprecipitated with anti-T1 reovirus antibody. During the first washing after immunoprecipitation the "unbound" fraction (supernatant) was collected for extraction of ssRNA. The "unbound" ssRNA was analyzed by electrophoresis on a 4.5% polyacrylamide gel and it was found that the amount of ssRNA which was not bound to antibody was the same in infected and uninfected cells (data not shown).

Fig. 8. Analysis of the interactions of the transfected M1D1 gene with viral proteins by radioimmunoprecipitation. Cytoplasmic extracts were analyzed by immunoprecipitation with anti-T1 reovirus ab followed by 10% PAGE. (1) reovirus infected, M1D1 (+) ssRNA transfected L929 cells; (2) (control) reovirus infected L929 cells; (3) M1D1 (+) ssRNA transfected control, uninfected L929 cells; (4) (control) L929 cells on which M1D1 (+) ssRNA was applied; cells were then washed. The positions of reovirus proteins and M1D1 (+) ssRNA are indicated.


1
2
3
4
λ1–λ3
μ1
μ1C
σ1
σ2
σ3

The beads containing "bound" ssRNA were washed with RIPA washing buffer and TE, followed by analysis on a 10% polyacrylamide gel for protein content and 4.5% polyacrylamide gel containing 8 M urea for ssRNA content. The detection of RNA and viral proteins in the same fraction would indicate that there was an interaction between transfected RNA and viral proteins. Both ssRNA and protein gels showed the presence of RNA associated with proteins, but the association was not specific since it was detected in both uninfected and infected cell lysates (Fig. 8). The experiment was modified as follows in attempts to detect specific RNA-protein interactions:

(i) the timing of infection and transfection was changed in order to introduce RNA early in infection, when protein-ssRNA complexes are normally being formed during reovirus infection (90). ^{32}P-labeled RNA was lipofected into virus infected cells 2-6 h after infection and cell lysates were harvested 18 h after transfection. L929 cells were infected at a high MOI of 50 with T1P2 to assure a one step infection.

(ii) a different lysis buffer containing DOC detergent in addition to Triton X-100 was used to extract the cytoplasmic fraction (90). This stronger detergent had been used to obtain reovirus particles at different stages of assembly from all structures extracted from the cytoplasm of infected cells.

(iii) the 2-methyl cap analog, which is more similar to authentic reoviral cap structures, was used in the transcription reaction instead of the 1-methyl cap analog in order to facilitate the recognition of the synthetic RNA by reoviral replicase.

(iv) immunoprecipitation was performed in TMN / 0.1% Triton X-100 buffer instead of RIPA buffers in order to detect weak RNA-protein interactions (90).

These changes did not affect the results. The RNA could still be recovered from both infected and uninfected cell lysates immunoprecipitated with anti-T1 reovirus ab indicating that the interactions between synthetic RNA and antibodies and/or proteins was not specific. The same result was obtained for M1D1 ssRNA as well as for M1T1 and T3 transcripts with anti-T1 reovirus ab and anti-T1μ2 ab (Fig. 9).

The transfected RNA did not appear to be specifically associated with viral proteins isolated by RIPA. Another explanation of this result would be that the transfected ssRNA was associated with ribosomes, was translated, remained associated with the μ2 translation product and therefore could be precipitated with anti-μ2 ab from both infected and uninfected cell lysates. However, the fact that the anti-T1 reovirus antibody has a negligible ability to precipitate the μ2 protein argues against this hypothesis.

It was shown previously (90) that the monoclonal antibodies; anti-σ3 + μ1C + μ1, anti-μNS and anti-σNS, precipitate protein-ssRNA complexes from reovirus infected cells. These monoclonal antibodies should be useful in studying interactions between ss synthetic RNA and viral protein complexes. An experiment to show the association of transfected RNA with viral proteins using anti-σ3 + μ1C + μ1, anti-μNS and anti-σNS monoclonal antibodies did not detect RNA-protein interactions (data not shown).

4.4.2 Separation of the cytoplasmic components of reovirus infected cells transfected with synthetic viral RNA by sucrose gradient centrifugation

Although the previous experiments could not detect synthetic RNA/protein interactions, it was possible that such complexes were formed but were not stable enough to remain associated during RIPA. Centrifugation of cell extracts through sucrose gradients was

used to assess and characterize protein interactions with transfected synthetic RNA. The cell lysates from reovirus infected, ^{32}P RNA transfected cells were separated on 4-40% continuous sucrose gradients. These centrifugation conditions should allow the detection of RNA associated with single proteins or small protein complexes. Using 15-40% sucrose gradients, association of transfected RNA with complete reovirus particles or subviral particles could also be assessed.

Following sucrose gradient centrifugation both protein content and transfected RNA distribution were examined by SDS PAGE. Transfected labeled RNA was not found to be associated with the fractions from the 4-40% sucrose gradient (Fig. 10). However, when M1D1 ssRNA extracted from the 15-40% sucrose gradient fractions was analyzed by PAGE, it was observed that the migration of synthetic RNA extracted from the cell lysate was different than for free M1D1 ssRNA (fractions # 6, 7 ,8 versus fractions # 7, 8, data not shown), but no difference was seen between RNA from lysates of infected and uninfected cells. This result indicated that the transfected RNA was associated with proteins which decreased its mobility in the gradient, however, cellular not viral proteins probably took part in this interaction. The amount of transfected ^{32}P RNA in the fractions from the 15-40% sucrose gradient which was harvested from infected and uninfected cells was measured by scintillation counting confirming the results from PAGE (Fig. 11).

Fig. 9. Interactions of transfected T1, T3 and M1D1 genes with viral proteins. (1) product of T1 transcription; (2-4) reovirus infected cells transfected with T1 synthetic gene; (5-7) control uninfected cells transfected with T1 synthetic gene; (8-10) reovirus infected cells transfected with T3 synthetic gene; (8-10) control uninfected cells transfected with T3 synthetic gene; (15-17) reovirus infected cells transfected with M1D1 synthetic gene; (12, 18,19) control uninfected cells transfected with M1D1 synthetic gene; (2, 5, 8, 11, 15, 18) lysates immunoprecipitated with anti-T1 reovirus ab; (3, 6, 9, 12, 13, 16) lysates immunoprecipitated with anti μ2-T1 ab; (4, 7, 10, 14, 17, 19) lysates immunoprecipitated with anti (A/FM/1/47) ab. Arrows indicate positions of full length M1 ssRNA and M1D1 ssRNA.

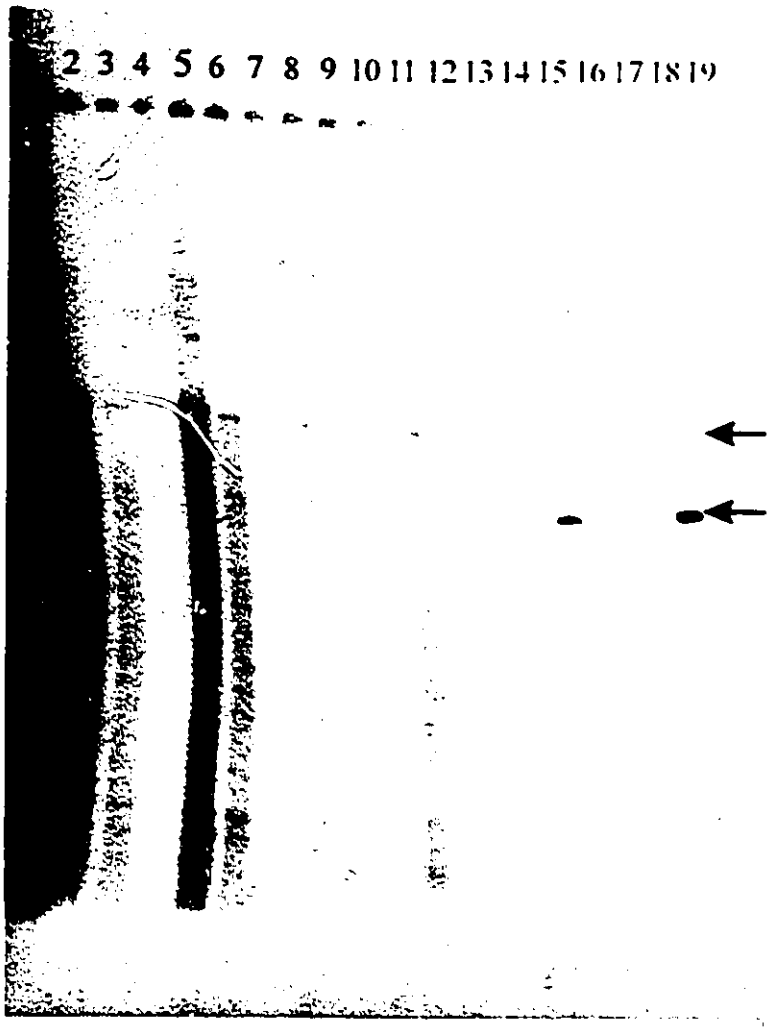

2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 10. Interactions of synthetic transfected M1T1 gene with viral components in infected cells after fractionation in 4-40% sucrose gradients. (1) T1 ssRNA transcription product; (2) bottom fraction after separation of cell lysates from reovirus infected cells transfected with T1 ssRNA; (3-11) fractions from the bottom to the top of the gradient for cell lysates from reovirus infected cells transfected with T1M1 (+) ssRNA; (12-19) fractions from the bottom to the top of the gradient for cell lysates from control, uninfected cells transfected with T1M1 (+) ssRNA. Positions of ^{32}P (+) ssRNA (arrow)and viral proteins are indicated.

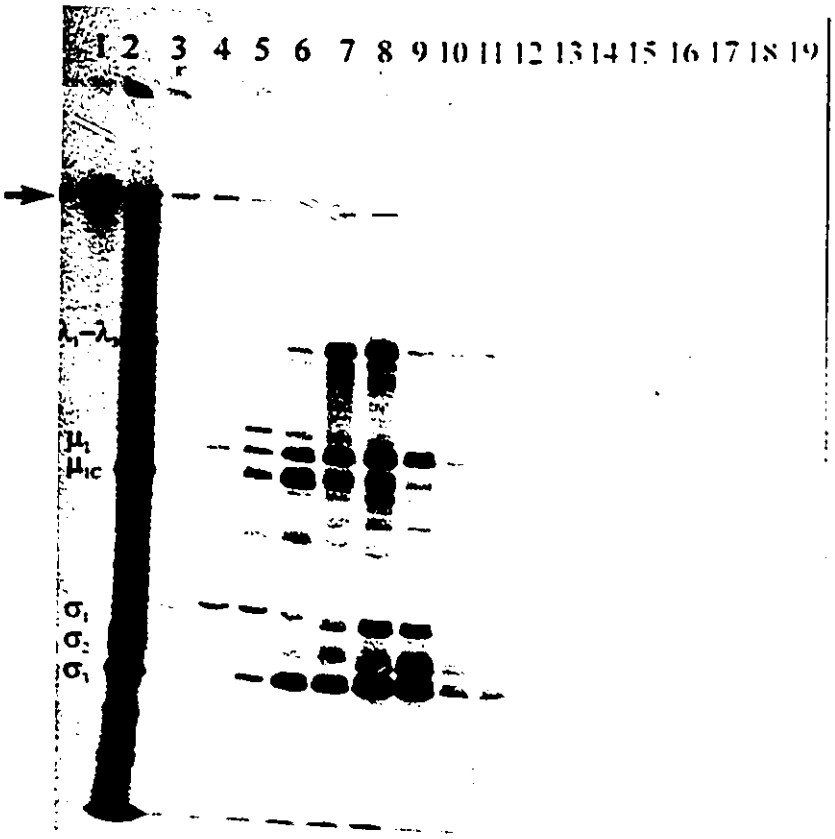
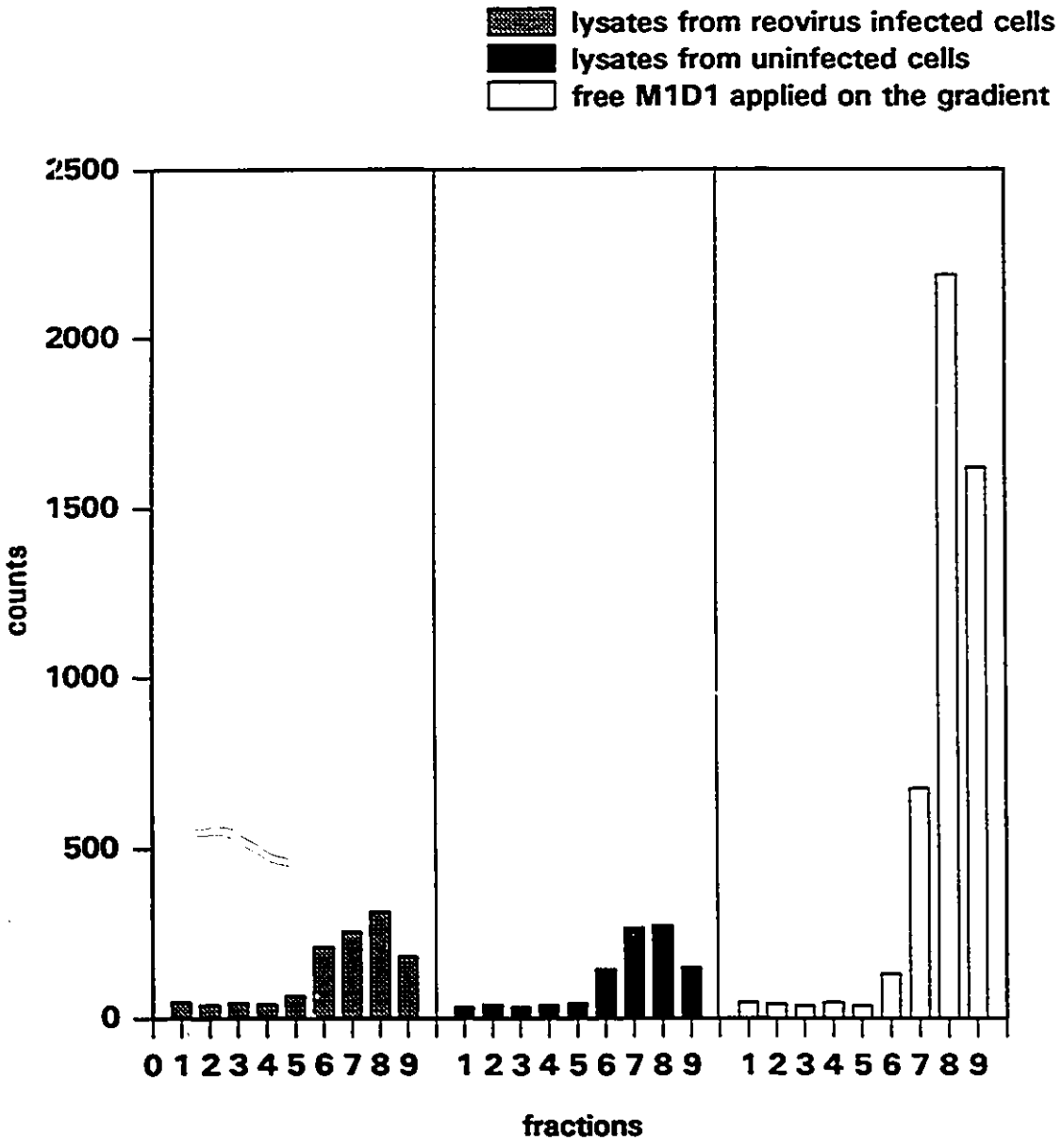

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
λ1–λ4
μ1
μ1c
σ1
σ2
σ3

Fig. 11. The position of synthetic M1D1 ssRNA in lysates of transfected cells, fractionated on 15-40% sucrose gradients. (1-9) fractions from the gradient from the bottom to the top; gray bars = reovirus infected cells; black bars = control, uninfected cells; white bars = free M1D1 ssRNA applied on the gradient.


lysates from reovirus infected cells
lysates from uninfected cells
free M1D1 applied on the gradient
2500
2000
1500
1000
500
0
counts
0 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
fractions

4.4.3 Analysis of reoviral ss and dsRNA by sucrose gradient centrifugation and radioimmunoprecipitation

The mobility of transfected synthetic RNA in the sucrose gradient was known from previous experiments. It was of interest to compare it with the migration of native, reoviral RNA to examine if synthetic and native RNA from reovirus infected cells sediment similarly or differently. Reovirus infected cells were metabolically labeled with ^{32}P in the presence of actinomycin D. The cytoplasmic fraction was extracted 24 h post infection and loaded onto a 15-40% sucrose gradient. Eight fractions were taken from the gradient, RNA was purified from them and then analyzed by PAGE.

Gels showed that the dsRNA migrates to the bottom fractions in the gradient (Fig. 13), whereas ssRNA is distributed throughout the gradient, peaking in fractions #5 and #6 (Fig. 12). The detection of dsRNA in the top fraction in the gradient (fraction #9, Fig. 13) may be explained by contamination of the last top fraction with the pellet. The distribution of native ssRNA was different from that of the transfected synthetic RNA (see 4.4.2). However, it is difficult to draw any conclusions from comparison of these results since transfected RNA represents only one viral gene and viral RNA represents all ten genome segments.

Half of the infected cell lysate from a 60 mm dish underwent immunoprecipitation with anti-T1 reovirus ab (Fig. 13 lane #1). This experiment was performed to examine the ability of anti-T1 reovirus ab to precipitate reoviral ssRNA, and showed that it predominantly precipitates dsRNA-containing complexes. There was little reoviral ssRNA bound to reovirus proteins that could be detected following RIPA with anti-T1 reovirus ab.

Fig. 12. Analysis of reoviral ssRNA by sucrose gradient (15-40%) centrifugation and electrophoresis on 4.5% polyacrylamide gels. (1-8) fractions from sucrose gradient centrifugation from the bottom to the top; (9) the pellet.


1 2 3 4 5 6 7 8 9

Fig. 13. Analysis of reoviral dsRNA by sucrose gradient (15-40%) centrifugation and electrophoresis on 7.5% polyacrylamide gels. (1) viral ss and dsRNA radioimmunoprecipitated with anti-T1 reovirus ab performed on unfractionated infected cell lysate; (2-9) fractions from a sucrose gradient from the bottom to the top, (10) the pellet.


1 2 3 4 5 6 7 8 9 10

4.4.4 Analysis of transfected M1D1 gene in sucrose gradient fractions by Northern blot hybridization

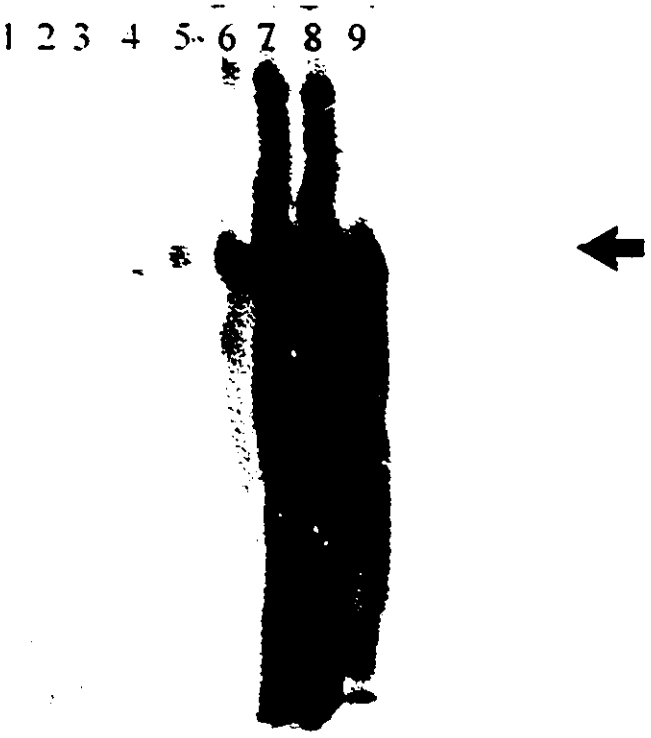
The distribution of reoviral ssRNA in 15-40% sucrose gradients is very broad (4.4.3). However, it was hypothesized that the distribution of M1 ssRNA might be more localized to one or two fractions. If so it should be easier to compare the position of transfected and native M1 genes in gradients. In order to distinguish the synthetic and native M1 genes, the deleted M1 gene was used for transfection. To overcome the experimental difficulty of accurately comparing two separate gradients, the synthetic and native genes were analyzed in one gradient by Northern blotting.

L929 cells were infected with T1P2 at an MOI of 50, 1 h prior to transfection. M1D1 (+) sense ^{32}P transcript was lipofected into infected cells. The cells were harvested 5 h post infection and applied to a 15-40% sucrose gradient.

EtBr staining of the PA gel showed that the native reoviral ssRNA was mainly in fractions #6 and #7, different than in experiment 4.4.2 and 4.4.3 probably due to the earlier time of virus harvesting from the cell culture (24 h versus 5 h). Autoradiography showed that the deleted synthetic ssRNA comigrated with the bulk of native ssRNA in fractions #6 and #7 (Fig. 14).

To find out if synthetic M1D1 comigrates with ssRNA of native M1 the ssRNA was transferred onto nylon membrane and hybridized with M1 specific primers, M15'(+) and M15'(-) (see Appendix I) complementary to the 3' end of the (+) and (-) strands of both M1 and M1D1 ssRNA. The hybridization allowed for visualization of the viral and synthetic genes at the same time.

Fig. 14. Analysis of M1D1 (+) ssRNA in transfected cell lysates 5 h post infection by 15-40% sucrose gradient centrifugation and electrophoresis on 4.5% polyacrylamide gel. (1) the pellet from the gradient centrifugation of reovirus infected L929 cells lysates transfected with M1D1 transcripts; (2-9) fractions from the bottom to the top from the gradient centrifugation of lysates from reovirus infected L929 cells transfected with M1D1 transcripts. The position of M1D1 is indicated by the arrow.


1 2 3 4 5 6 7 8 9

The comigration of the ssRNA of native M1 and synthetic M1D1 into the same fraction of the sucrose gradient could mean functional and structural similarity. M1D1 was indeed detected in the same fraction as the M1 gene, namely fraction #6 and #7 (Fig. 15).

To explain these results, it is proposed that early in infection the reoviral M1 ssRNA and the synthetic gene are both associated with polysomes and not with reoviral structures. It seems that synthetic and native M1 genes are equally recognized by the cellular machinery. This would explain the similar migration of synthetic RNA transfected into infected and uninfected cells in sucrose gradients which is different from free RNA. Additional experiments have to be performed to confirm this hypothesis.

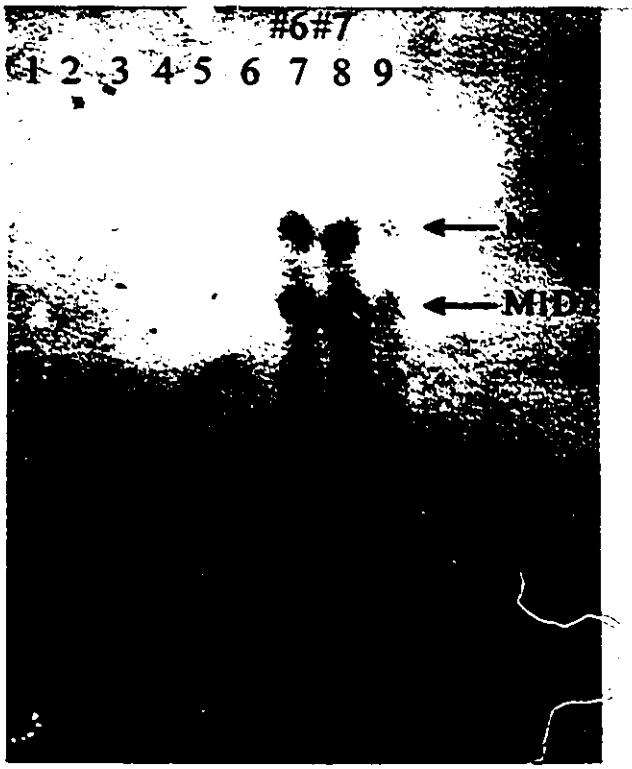
4.5 Selection of lethally mutated reovirus

Attempts to obtain a lethal M1 mutant by serial undiluted passage in $\mu 2$ expressing cells and UV mutagenesis were unsuccessful but are presented in Appendix II

4.6 Introduction of synthetic reoviral ssRNA into open-core particles

The major obstacle to the introduction of synthetic RNA into reovirus is that replication takes place inside the reovirus particle during assembly. This difficulty can be overcome by "opening" the reovirus particle. Synthetic ssRNA would then have access to the reoviral replicase. It was shown that rotavirus single shelled cores treated by low-ionic strength buffer open to release dsRNA and are then able to replicate exogenously supplied synthetic rotaviral RNA (90). Experiments were performed to examine if this method could be feasible for the introduction of synthetic ssRNA into reovirus particles.

Fig. 15. Northern blot hybridization of transfected M1D1 (+) ssRNA to correlate its position with viral M1 ssRNA in sucrose gradient fractions. (1) the pellet from the gradient centrifugation of reovirus infected L929 cell lysates transfected with M1D1 transcripts; (2-9) fractions from the bottom to the top from gradient centrifugation of reovirus infected L929 cell lysates transfected with M1D1 transcripts. Arrows indicate positions of M1 and M1D1.


#6#7
1 2 3 4 5 6 7 8 9
MID

4.6.1 "Opening" reoviral particles and replication activity

To "open" reovirus particles, cores were dialyzed O/N in low ionic strength buffer. To examine the "openness", and thus the exposure of the dsRNA genome, the dialyzed cores were treated with 50 μg/ml RNase A and electrophoresed on a 7.5% polyacrylamide gels. Unlike rotavirus, reovirus dsRNA from dialyzed cores was resistant to RNase A treatment, indicating that cores were not "open" (Fig. 16B).

Various mechanical and chemical treatments were tested in order to "open" the core particle (Fig. 2). These treatments were followed by RNase A digestion and SDS PAGE to assess the structural integrity of particle.

Resistance of dsRNA to RNase A digestion was shown following:

(1) treatment with urea at concentrations up to 4.8 M (Fig. 16B),

(2) treatment with formamide at concentration up to 20% (Fig. 16B),

(3) treatment of cores with 1% DOC (Fig. 16A),

(4) treatment of cores with pH 10.5 (not shown),

(5) treatment of cores with 10% 2ME (not shown),

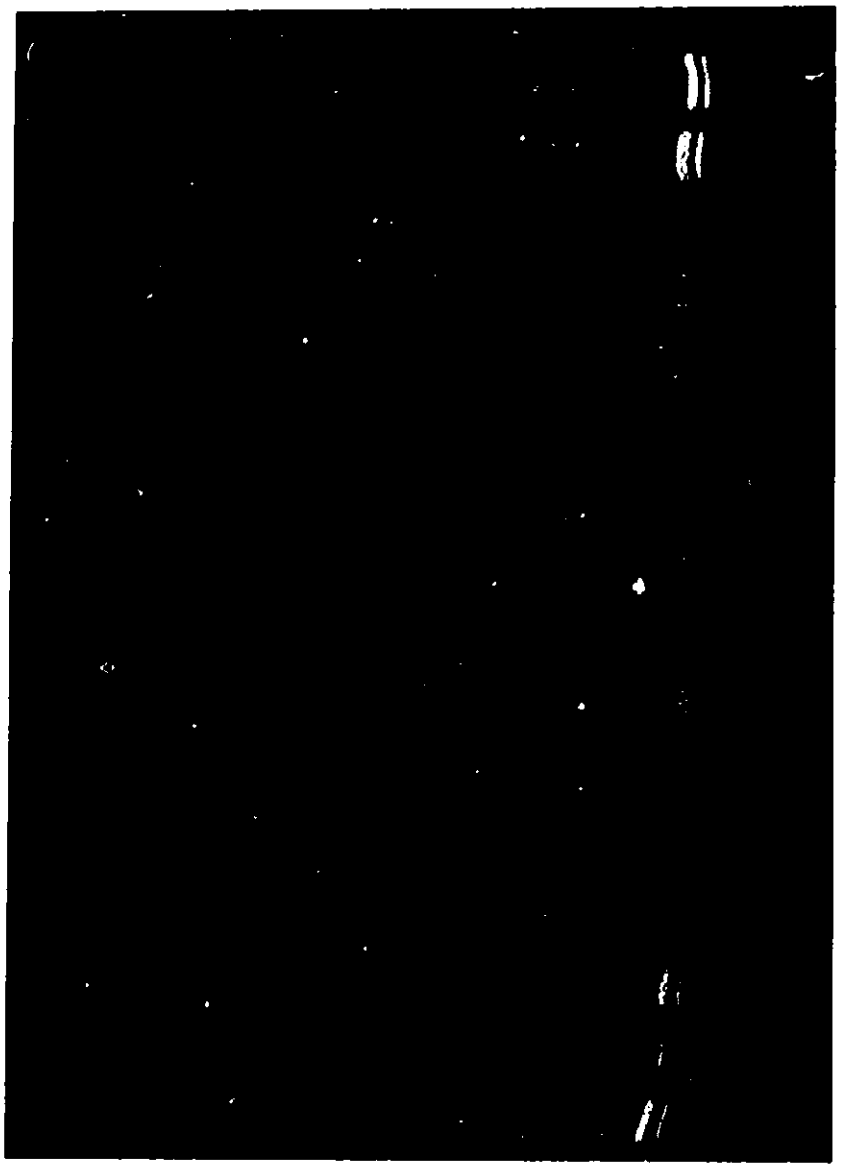
(6) treatment of cores with 250 mM EDTA (not shown),

(7) 6 cycles of freeze/thaw of dialyzed cores (Fig. 16B),

(8) heating of virus and core for 1 min. at 60°C (Fig. 16B),

(9) and 1-12 min. sonication of dialyzed cores (not shown).

Fig. 16. Characterization of treated reovirus particles by RNase A digestion. Reovirus was subjected to various treatments as indicated above each lane. EtBr stained 7.5% polyacrylamide gels of dsRNA of treated reovirus particles after : (A) 50μg/ml and (B) 100μg/ml RNase A digestion for 30 min. at 37°C. Untreated reovirus particles are included for each RNase A digestion as a control.



-1%DOC/RNase A

-1%DOC

-4M guanidine/RNase A

-4M guanidine

-sat.urea 60° C/RNase A

-sat.urea 60° C

-sat.urea 40° C/RNase A

-sat.urea 40° C

-sat.urea RT/RNase A

-sat.urea RT

-core/50% formamide/RNase A

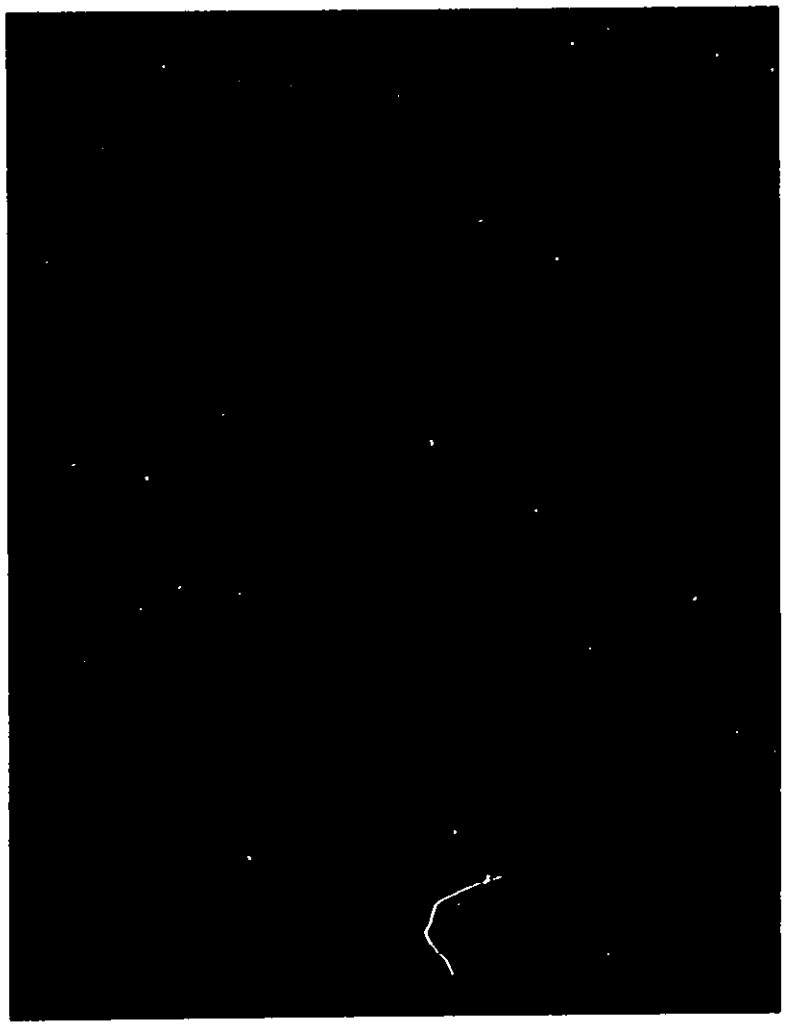
-core/50% formamide

-virus/50% formamide/RNase A

-virus/50% formamide

-core

-virus



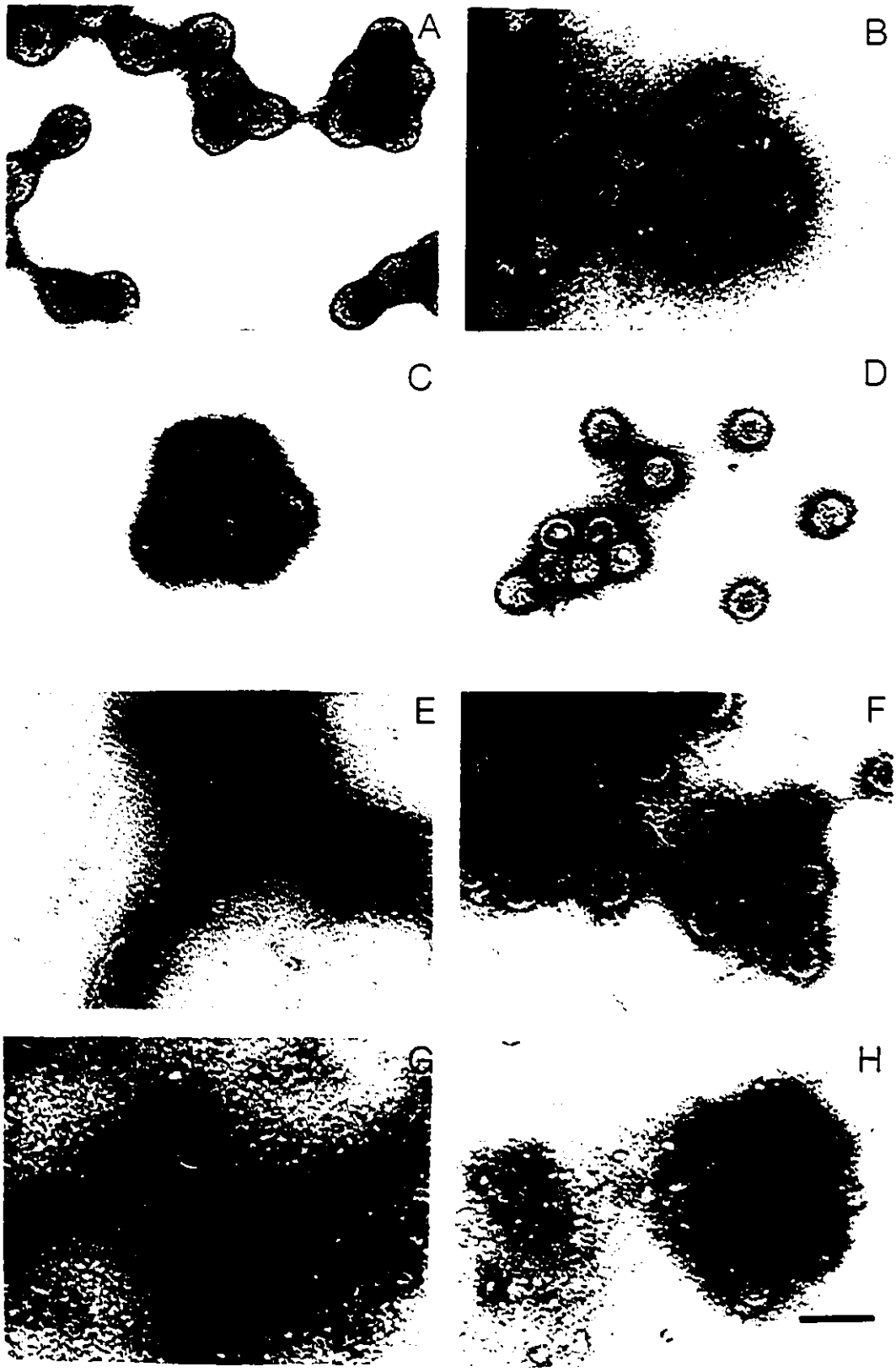
-dial. core
-dial. core/RNase A
-20% formamide
-20% formamide/RNase A
-30% formamide
-30% formamide/RNase A
-sat. urea
-sat. urea/RNase A
-4.8M urea
-4.8M urea/RNase A
-1.6M urea
-1.6M urea/RNase A
-6x freeze/thaw
-6x freeze/thaw/RNase A
-dial. core 60° C
-dial. core 60° C/RNase A
-virus 60° C
-virus 60° C/RNase A
-core 60° C
-core 60° C/RNase A

B

The following treatments rendered dsRNA susceptible to RNase A: saturated urea and 4 M guanidine treatments of cores (Fig. 16A), and 30% and 50% formamide treatments of virions and cores (Fig. 16A,B). Surprisingly the susceptibility of dsRNA to RNase A was greater for cores treated with saturated urea at 40°C and less for cores treated at 60°C. The dsRNA of 50% formamide treated cores was more susceptible than that of virion dsRNA (Fig. 16A). Cores treated with 0.8 M, 2.4 M and 4.8 M urea were resistant to 500 μg/ml of RNase A. The results of these experiments are summarized in Table 2.

Electron Microscopy (EM) studies were undertaken to examine the structure of reovirus after various treatments (Table 2). The presence of "open" particles was expected in treatments where dsRNA was RNase A susceptible. However, EM revealed a lack of any particles after treatment with saturated urea, and a small number of damaged, but not "open", particles after 50% formamide treatment (Fig. 17 E,G,H). The particles were observed only if the samples treated with 50% formamide were diluted 6x prior to dialysis, resulting in a shorter time of exposure to formamide. It was also observed that with an increase in the concentration of either urea or formamide, from 1.6 M urea to saturated urea or from 20% to 50% formamide, smaller numbers of particles were observed. This was probably due to disruption and/or clumping of particles. Heating (Fig. 17 D), sonication, 20% formamide, 4.8 M urea, pH 10.5, 250 mM EDTA and dialysis did not result in any alteration of core particle structure discernable by EM (not shown).

Fig. 17. Electron micrographs of untreated and treated virus particles; (magnification x 120000); (A) virions; (B) cores; (C) virions/60°C, 1 min.; (D) cores/60°C, 1 min.; (E) virions/50% formamide; (F) dialyzed cores/6xfreeze/thaw; (G) cores/saturated urea; (H) cores/50% formamide. Bar 100 nm.


A
B
C
D
E
F
G
H

Heat treatment resulted in the appearance of protrusions on the surface of the virus particles (Fig. 17C). Another interesting finding was that dialyzed cores subjected to 6 cycles of freeze/thaw were altered in such a way as to be permeable to dye, with the appearance of "empty" particles and were generally misshapen (Fig. 17F). The relationship of these alterations in the structure of reovirus particles to transcriptional activity was subsequently examined (see section 4.6.2).

In spite of the lack of "open" particles, replication assays were performed with dialyzed cores, with cores treated with 50%, 40%, 30%, 20% formamide, saturated, 4.8 M, 3.2 M and 1.6 M urea and with dialyzed cores that had been sonicated for 1, 2, 4, 8, 12 min. (Table 2).

The M1D1 construct was used as a synthetic gene in this study (see 4.2). M1D1 DNA was prepared for run off transcription by Bsm I digestion and T4 polymerase treatment. Two kinds of experiments were performed. In the first series of experiments, the M1D1 ssRNA was ^{32}P labeled and in the second series of experiments the M1D1 transcript was not labeled but [$\alpha^{32}P$]UTP was added to the replication reaction to detect the dsRNA product. A problem in the experiments with ^{32}P M1D1 ssRNA was that false positive results (ds^{32}P labeled RNA) were obtained due to synthesis of the second strand on T7 transcripts, resulting in dsRNA. Presumably this was caused by inefficient T4 polymerase repair of the 3' end of DNA and the priming of T7 RNA polymerase on the (-) 5' end of the DNA template. In experiments where [$\alpha^{32}P$]UTP was added to the replication assay, the label was incorporated into native ss transcripts rather than dsRNA due to viral transcription. In neither case was dsRNA replicated on the synthetic M1D1 ssRNA transcription products or native ssRNA (data not shown).

4.6.2 Transcriptional activity of cores following different treatments

In the replication assay it was observed that the different treatments of virus and cores affected transcriptase activity. Different transcriptional activity indicates that structural changes of particles and/or activation/inactivation of viral polymerase has occurred. In order to study this effect, transcription assays were performed at two temperatures, 34°C and 50°C. The same buffer and protocol were used as for the replication assay (see 4.6.1). The transcription assay was performed in the presence of [α^{32}P] UTP and labeled products were analyzed by electrophoresis on 4.5% polyacrylamide gels containing 8 M urea. The experiments showed that there were differences in the activity of the treated particles at different temperatures.

At 34°C, the following treatments resulted in particles that were transcriptionally more active than untreated cores:

(1) heating of cores at 60°C for 1 min.,

(2) heating of dialyzed cores at 60°C for 1 min.,

(3) treatment of cores at pH 10.5 (Fig. 18).

Decreased transcriptional activity relative to native cores at 34°C was observed with the following treatments:

(1) 2, 8 and 12 min. sonication of dialyzed cores,

(2) 6x freeze/thaw of dialyzed cores,

(3) treatment of cores with 10% 2ME,

(4) treatment of cores with 1.6 M and 4.8 M urea and

(5) heating of virions for 1 min. at 60°C (Fig. 18).

Dialysis of cores in low ionic strength buffer and 20% formamide treatment did not affect the transcriptional activity (Fig. 18). However, 20% formamide, 1.6 M and 4.8 M urea treatments sometimes resulted in particles of much higher transcriptional activity relative to cores at 34°C (data not shown). The reason for these differences in the transcriptional activity of cores treated with 20% formamide, 1.6 M and 4.8 M urea will have to be further examined.

Formamide at 30%, saturated urea and 250 mM EDTA abolished transcriptase activity at both 34°C and at 50°C (Fig. 18, 19) indicating complete transcriptional inactivation by these treatments.

Transcriptional assays of cores prepared at pH 10.5, heat treated cores and heat treated dialyzed cores at 50°C showed more transcription products than untreated cores (Fig. 19). However, interestingly dialyzed cores, dialyzed cores sonicated for 2, 8 and 12 min. and virions treated at 60°C for 1 min. were more active than untreated cores at 50°C transcriptional assay, whereas 20% formamide treated cores were less active, which is opposite to what was seen at 34°C (Fig. 19). The activity of 1.6 M and 4.8 M urea treated cores at 50°C was greater than at 34°C and approximately the same as native cores or a little greater.

Fig. 18. The effect of chemical and physical treatments of cores and virions on transcriptase activity at 34°C. Data from 3 independent experiments were analyzed densitometrically. A value of 1 was assign to cores, and data for treated cores were calculated accordingly. (1) native cores; (2) dialyzed cores; (3) cores/20% formamide; (4) cores/30% formamide; (5) cores/saturated urea; (6) cores/4.8 M urea; (7) cores/1.6 M urea; (8) cores/10% 2 ME; (9) dialyzed cores/2 min. sonication; (10) dialyzed cores/8 min. sonication; (11) dialyzed cores/12 min. sonication; (12) dialyzed cores/6xfreeze/thaw; (13) cores/pH 10.5; (14) dialyzed cores/60°C, 1 min.; (15) virions/60°C, 1 min.; (16) cores/60°C, 1 min.; (17) cores/250 mM EDTA; (18) cores/50% formamide; (19) water (negative control).

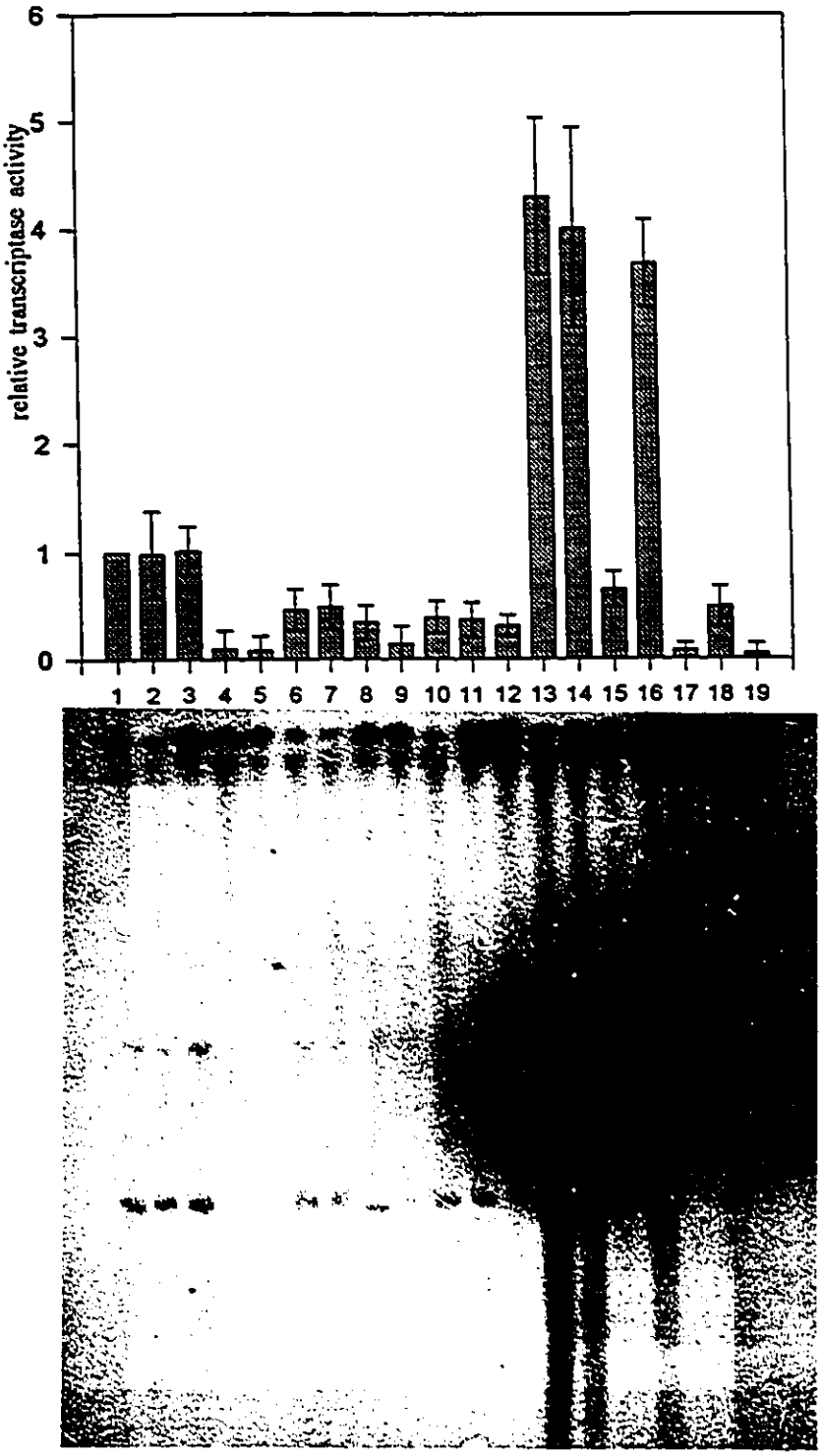
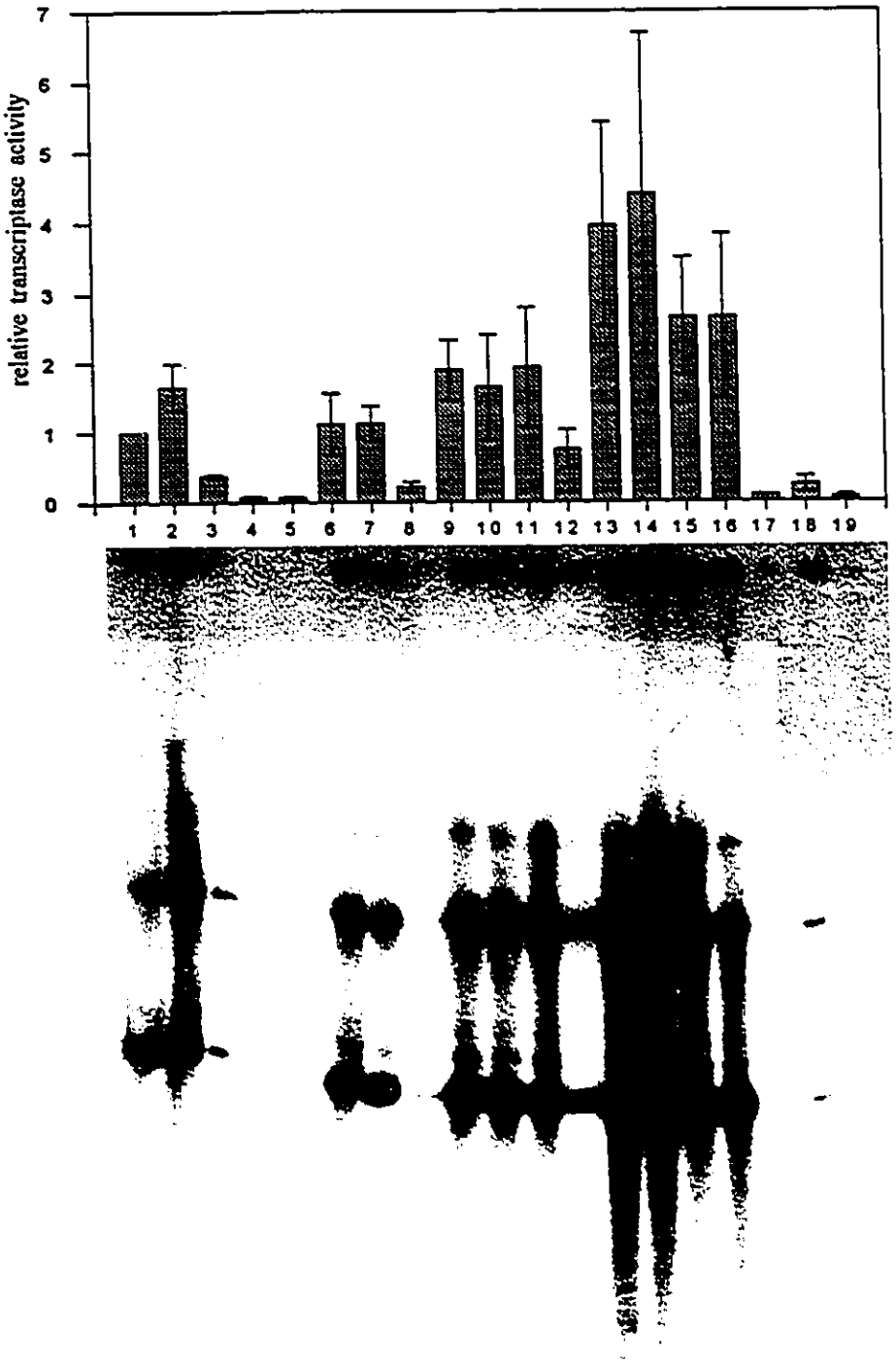

relative transcriptase activity
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Fig. 19. Transcriptional activity of treated cores and virions at 50°C. Transcriptional activity from 3 independent experiments was analyzed. [α^{32}P] UTP labeled bands were cut from 4.5% polyacrylamide gels and quantitated by liquid scintillation counting. A value of 1 was assign to cores, and data for treated cores were calculated accordingly. (1) native cores; (2) dialyzed cores; (3) cores/20% formamide; (4) cores/30% formamide; (5) cores/saturated urea; (6) cores/4.8 M urea; (7) cores/1.6 M urea; (8) cores/10% 2 ME; (9) dialyzed cores/2 min. sonication; (10) dialyzed cores/8 min. sonication; (11) dialyzed cores/12 min. sonication; (12) dialyzed cores/6xfreeze/thaw; (13) cores/pH 10.5; (14) dialyzed cores/60°C, 1 min.; (15) virions/60°C, 1 min.; (16) cores/60°C, 1 min.; (17) cores/250 mM EDTA; (18) cores/50% formamide; (19) water (negative control).


relative transcriptase activity
0
1
2
3
4
5
6
7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Dialyzed cores that were subjected to 6 cycles of freeze/thaw and 10% 2ME treated cores were less active at 50°C than untreated cores (the same as at 34°C) (Fig. 19).

Transcriptional activity of cores treated with 50% formamide (Fig. 18 lane 18 and Fig. 19 lane 18) was higher then in subsequent experiments. Virions and cores were treated with 50% formamide for 15 min. and then they were diluted 6x with STE buffer prior to dialysis. This may explain why this preparation retained some transcriptional activity. In later experiments, the formamide treated cores were not diluted prior to dialysis and at formamide concentration of 30% and higher, the cores were transcriptionally inert.

Figure 20 shows the ratio of transcriptional activity of treated cores and virions at 50°C versus 34°C, normalized to native cores (Table 2). The following treatments resulted in the same relative temperature dependent transcriptional activity as observed for native cores:

(1) cores treated with pH 10.5,

(2) heat treated cores,

(3) heat treated dialyzed cores and

(4) dialyzed cores

The 50°C/34°C ratio was higher for:

(1) cores treated with 1.6 M and 4.8 M

(2) 2, 8 and 12 min. sonicated dialyzed cores,

(3) virions heated for 1 min. and

(4) dialyzed cores following 6 freeze/thaw cycles,

suggesting differences in the transcriptase complexes.

The ratio was lower for cores treated with 20% formamide and 10% 2ME. Transcriptional activity was observed only when particles were detected by EM (Table 2). The only exceptions were cores treated with 250 mM EDTA. The presence of particles was detected by EM, however, the transcriptional activity was not observed. EDTA was not removed from cores before the transcription assay was performed, and its presence would be expected to inhibit the activity of transcriptase.

Fig. 20. Relative 50°C/34°C ratio of transcription . Note that the 50°C/34°C ratio for dialyzed cores, pH 10.5, dialyzed cores/60°C, 1 min., and cores/60°C, 1 min., was the same as for native cores. This ratio was lower for cores treated with 20% formamide and 10% 2ME relative to native cores, and higher for cores treated with 4.8 M and 1.6 M urea, for sonicated dialyzed cores, for dialyzed cores after 6 cycles of freeze/thaw and for virions heated for 1 min. at 60°C relative to native cores.

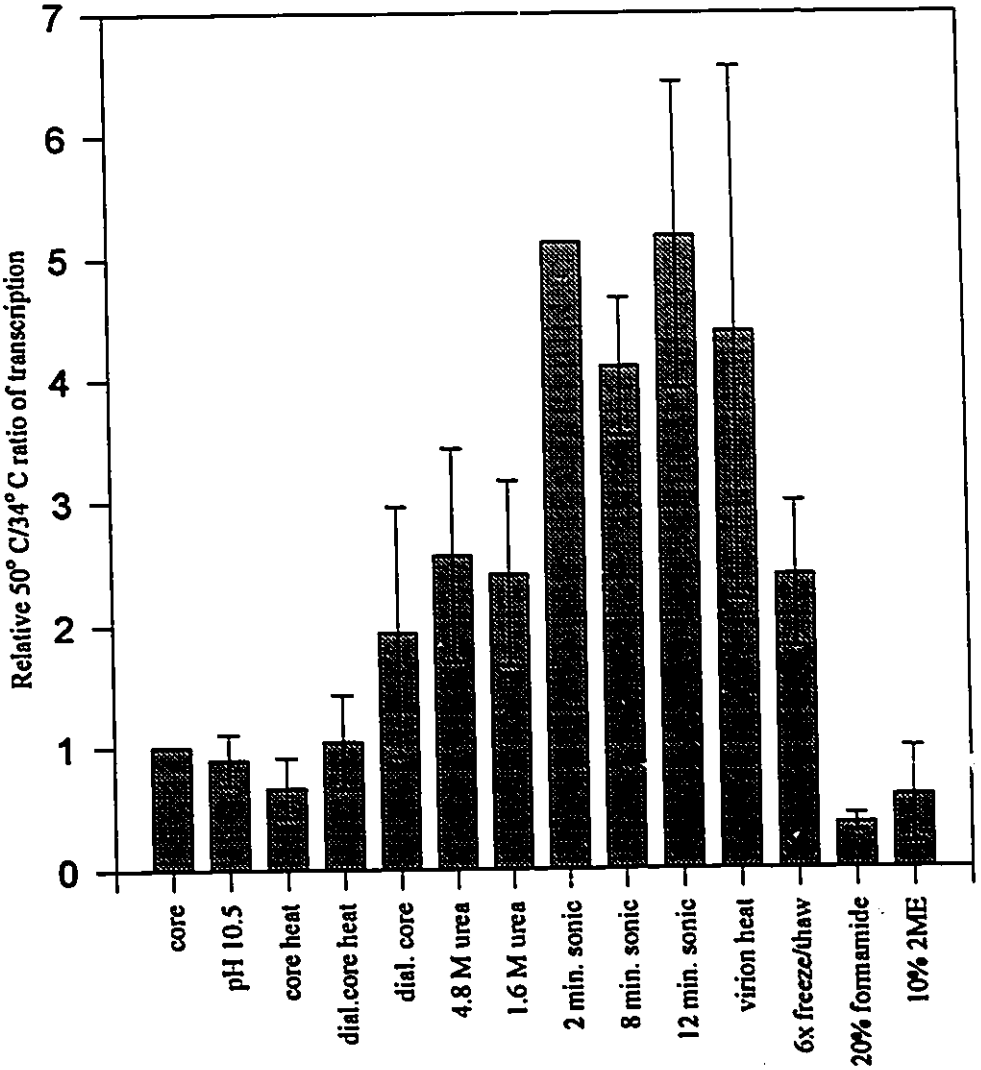

Relative 50° C/34° C ratio of transcription
0
1
2
3
4
5
6
7
core
pH 10.5
core heat
dial.core heat
dial. core
4.8 M urea
1.6 M urea
2 min. sonic
8 min. sonic
12 min. sonic
virion heat
6x freeze/thaw
20% formamide
10% 2ME

Table 2. Summary of the effects of different treatments on reovirus structure and transcriptional activity. (-) indicates RNase A resistance or lack of replicase activity, (✚) indicates RNase A susceptibility, (⇑) indicates an increase of transcriptional activity at 50°C/ 34°C relative to native cores, (⇓) indicates a decrease in the transcription ratio relative to native cores, (nd) not done.

Treatment	RNase A susceptibility	EM	Transcription ratio 50°C/ 34°C	Replication activity
250 ml M EDTA	-	no affect	inactivation	nd
4 M guanidine	✚	nd	nd	nd
10% 2 ME	-	nd	⇓	nd
1% DOC	-	nd	nd	nd
pH 10.5	-	no affect	unchanged	nd
core 60°C	-	no affect	unchanged	nd
virus 60°C	-	protrusions	⇑	nd
dial. core 60°C	-	no affect	unchanged	nd
dial. core	-	no affect	⇑	-
dial. core 6x freeze/thaw	-	dye permeable particles	⇑	nd
0.8 M urea	-	decreasing number of particles (clumping)	nd	nd
1.6 M urea	-		⇑	-
2.4 M urea	-		nd	nd
3.2 M urea	-		nd	-
4.8 M urea	-		⇑	-
sat. urea	✚	no particles	inactivation	-
20% formamide	-	less particles (clumping)	⇓	-
30% formamide	✚	no particles	inactivation	-
40% formamide	nd	nd	nd	-
50% formamide	✚	no particles	nd	-
2 min. sonication	-	no affect	⇑	-
4 min. sonication	-		nd	-
8 min. sonication	-		⇑	-
12 min. sonication	-		⇑	-

4.6.3 Purification of cores treated with 4.8 M urea and 12 min. sonication

To determine if the treatments resulted in structural changes of reovirus particles, the 4.8 M urea treated cores and dialyzed cores sonicated for 12 min. were subjected to centrifugation in CsCl gradients. These two treatments were chosen because both resulted in transcriptionally active cores with an increased 50°C/34°C ratio of transcriptional activity, suggesting that the transcriptase components were altered. Cores treated with 4.8 M urea had a density of 1.45 g/ml, whereas sonicated dialyzed cores formed a broad band at 1.36 g/ml in CsCl gradients. The comparison of the position of the bands in CsCl gradients is shown in Fig. 21. The changes in the density relative to the core position (1.44 g/ml) could be explained by the loss of protein in the case of 4.8 M urea treatment and loss of dsRNA in the case of sonication. Alternatively structural changes of particles could account for density differences.

Dialyzed cores provided two discrete bands in CsCl gradients (Fig. 21). The top and the bottom bands had the same density as native cores and 4.8 M urea treated cores, respectively indicating a heterologous population following low ionic strength dialysis. No differences between particles in these two bands were found by EM. Also both were more active than cores in the transcription assay and had a full complement dsRNA and core proteins (data not shown).

Six fractions were taken from each gradient including the band. The A_{260} was measured and it was found that 22% of sonicated particles and 17% of 4.8 M treated particles were lost during the CsCl gradient centrifugation by comparison with untreated native cores.

The content of these particles was examined on a 7.5% polyacrylamide gel. The gel was silver stained to visualize both dsRNA and proteins (Fig. 22). The gel showed that 4.8 M urea treated and sonicated cores contained all dsRNA segments and core proteins.

Additionally, dsRNA was detected in fraction #5 and #6 from 4.8 M urea treated cores indicating release of dsRNA from 4.8 M urea treated cores that accounts in part for the decreased yield after purification of 4.8 M urea treated cores. A faint band of λ3 protein associated with dsRNA in fraction #6 was detected by silver staining. The dsRNA from fraction #6 was susceptible to RNase A digestion (100 μg/ml) whereas the dsRNA from cores, cores treated with 4.8 M urea and sonicated dialyzed cores was resistant to RNase A (Fig. 23). The transcriptional activity was determined for 4.8 M urea and sonicated cores before and after banding in CsCl. Fraction #6 from 4.8 M urea treated cores was included as well as native cores, as controls. Transcriptional activity of 4.8 M urea and sonicated cores at both 34°C and 50°C was retained after banding (Fig. 24).

Most interesting however, was the observation that fraction #6 obtained from the top of the CsCl gradient of 4.8 M urea treated cores showed transcriptional activity at both 34°C and 50°C. Transcriptional activity of this preparation was not expected since the dsRNA was RNase A susceptible and because EM studies revealed no virus particles in this fraction but instead fibrillar structures that are probably dsRNA (Fig. 25). The presence of λ3 and dsRNA in fraction #6 indicated that this material may represent a minimal transcriptase complex obtained from core particles. More experiments have to be performed to confirm the presence of λ3 and to assess the presence of other proteins associated with this fraction.

Fig. 21. Separation of treated cores in 1.3-1.6g/ml CsCl gradients; (1) virions; (2) cores; (3) dialyzed cores; (4) 4.8 M urea treated cores; (5) 12 min. sonicated dialyzed cores.

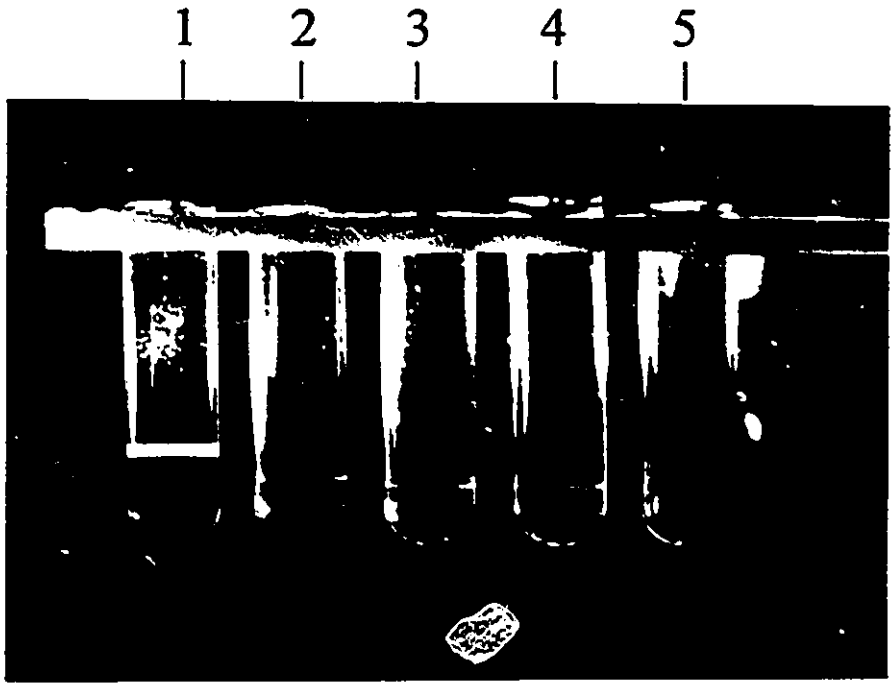

1
2
3
4
5

Fig. 22. Protein and dsRNA content of CsCl gradient fractions of cores, sonicated dialyzed cores and cores treated with urea; dsRNA and proteins were visualized by silver staining after electrophoresis of samples on 7.5% polyacrylamide gels. Numbers 1-6 refer to fractions from the bottom to the top taken from the CsCl gradients. Virions were included as a marker for reoviral dsRNA and proteins. The positions of reovirus dsRNA and proteins are indicated.

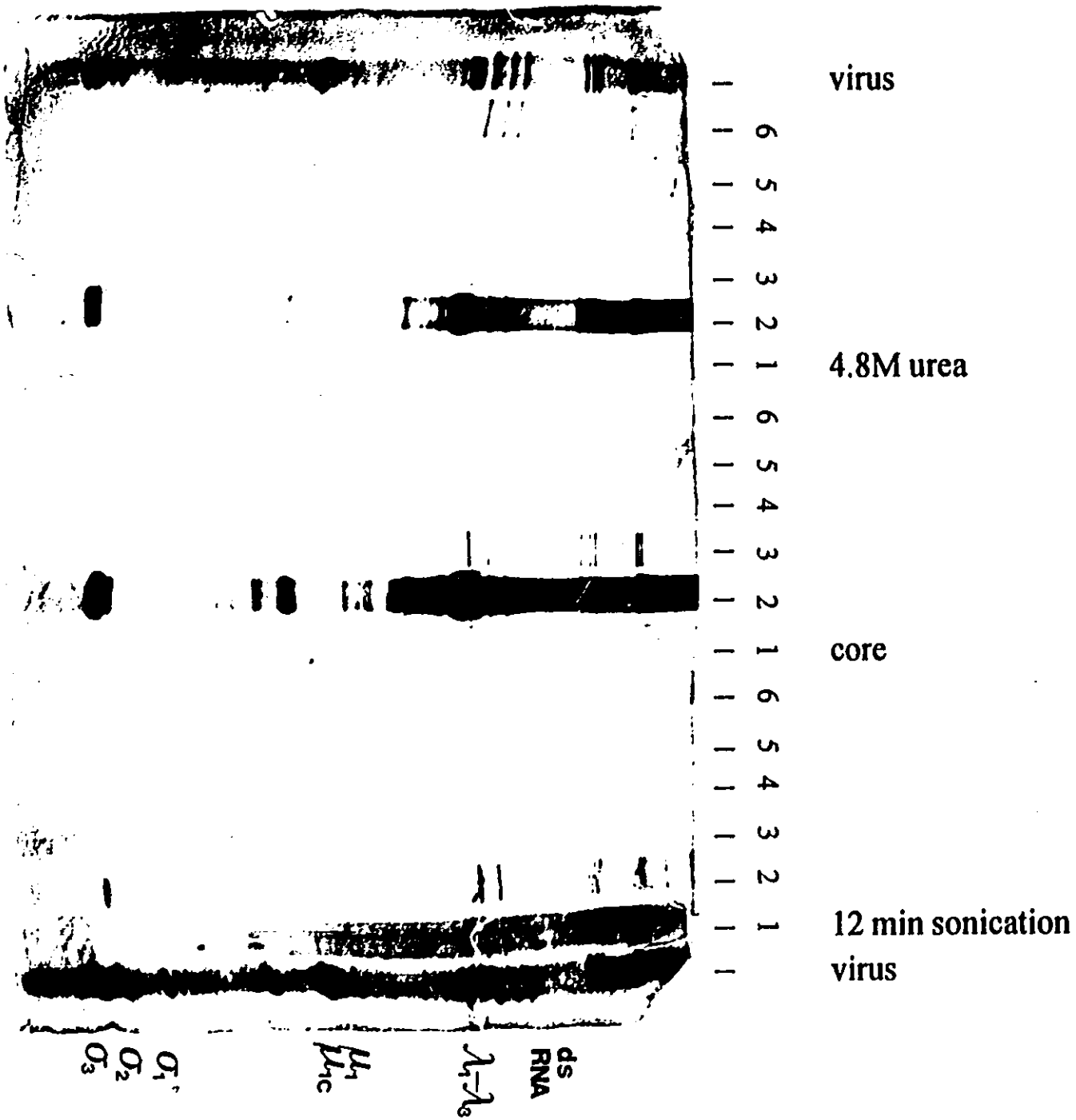

virus
6 5 4 3 2 1
4.8M urea
6 5 4 3 2 1
core
6 5 4 3 2 1
12 min sonication
virus
σ3 σ2 σ1
μ1c μ1
λ1–λ3
ds RNA

Fig. 23. RNase A susceptibility of treated reovirus following CsCl gradient purification. (1-2) cores; (3-4) 4.8 M urea treated cores; (5-6) fraction #6 obtained from 4.8 M urea treated cores; (7-8) 12 min. sonicated dialyzed cores. Cores and treated particles were incubated with 100 μg/ml RNase A at 37°C for 30 min. (+) RNase A treatment.

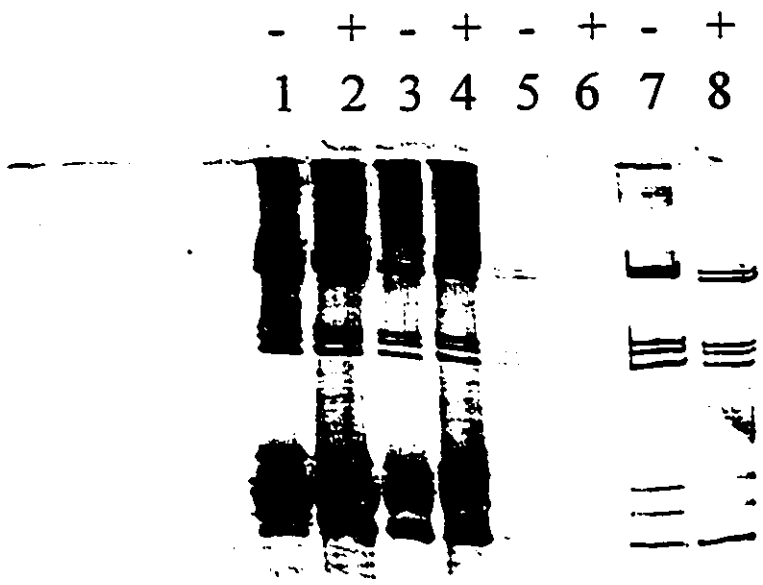

- + - + - + - +
1 2 3 4 5 6 7 8

Fig. 24. Transcriptional activity at 34°C and 50°C of cores treated with 4.8 M urea and sonicated dialyzed cores before and after purification on CsCl gradients. (1-7) transcriptional assay at 34°C; (8-14) transcriptional assay at 50°C; (1-3 and 8-10) reovirus before banding; (4-7 and 11-14) reovirus after banding.

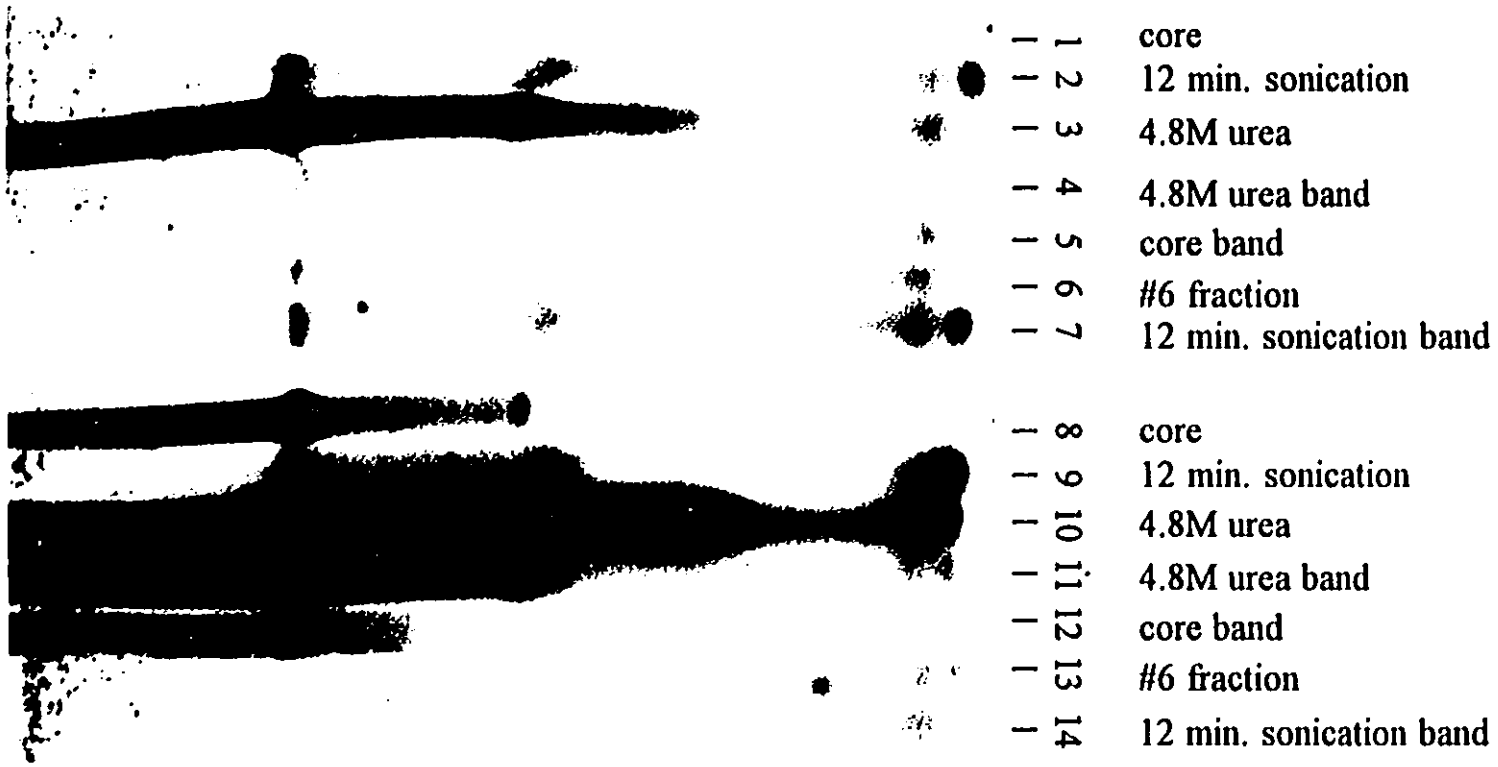
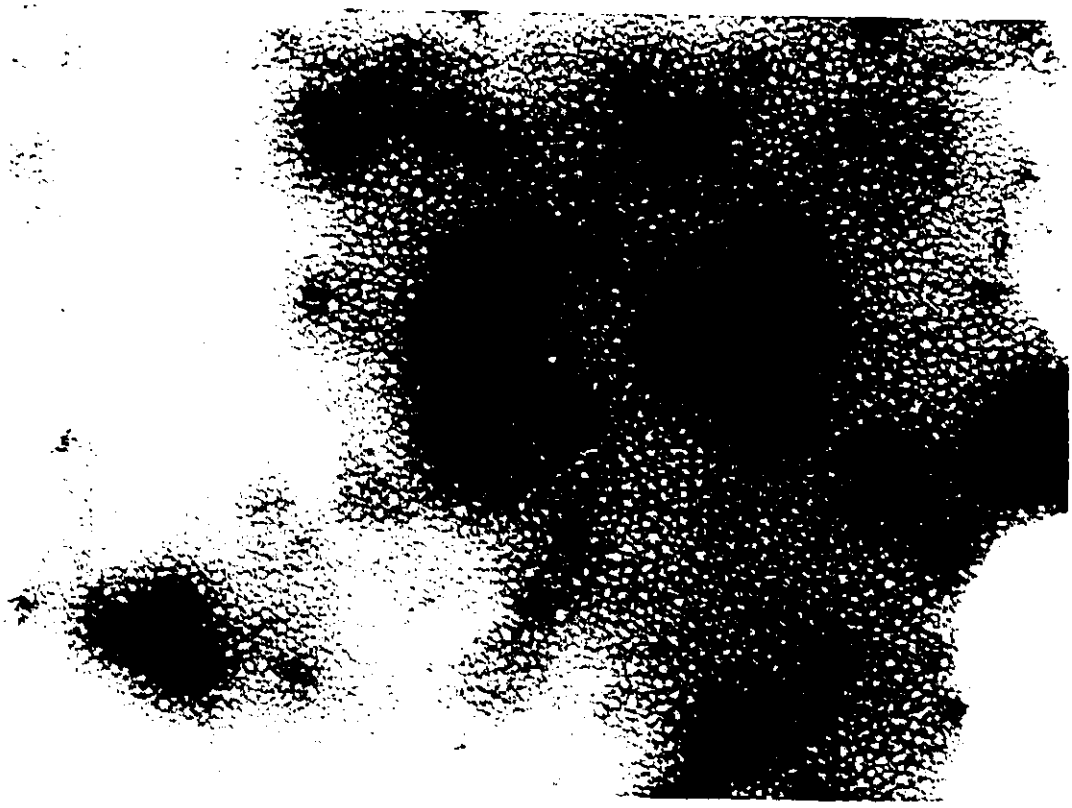

1 core
2 12 min. sonication
3 4.8M urea
4 4.8M urea band
5 core band
6 #6 fraction
7 12 min. sonication band
8 core
9 12 min. sonication
10 4.8M urea
11 4.8M urea band
12 core band
13 #6 fraction
14 12 min. sonication band

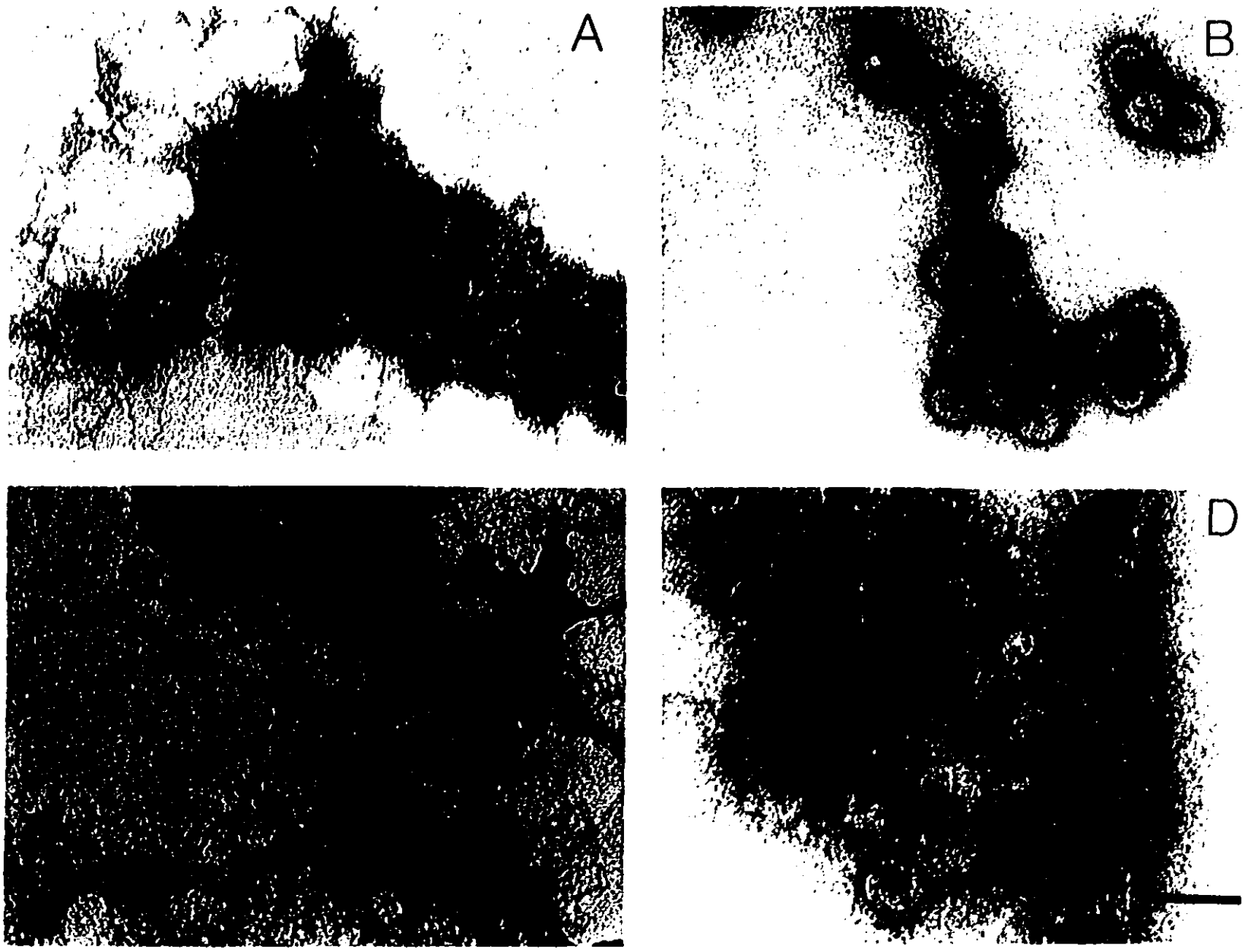
Fig. 25. Electron micrograph of transcriptase complex, "fraction #6". Fraction #6 obtained form purification of cores treated with 4.8 M urea on CsCl gradient was examined by electron microscopy. (magnification x 56 430)



The electron microscopy of both 4.8 M urea and sonicated cores purified on CsCl gradients showed generally intact particles and a small number of particles at different stages of disruption (Fig. 26). Cores treated with 4.8 M urea had some breaks and the core shell was not continuous (Fig. 26A) whereas sonication alone did not result in any major alteration of core particles that could be detected by EM (Fig. 26B). However, after additional sonication (1 min.), sonicated particles seem to be disrupted into smaller subunits that were still loosely bound together (Fig. 26D). The presence of "strands" was detected after a second sonication step for both sonicated dialyzed cores and 4.8 M urea treated cores (Fig. 26C, 26D). The dsRNA after second sonication of sonicated dialyzed cores was resistant to RNase A possibly due to the intertwining of dsRNA (data not shown). The second sonication resulted in a large decrease in the transcriptional activity of purified 4.8M urea treated cores and transcriptional inactivation of purified 12 min. sonicated dialyzed cores (data not shown).

"Open core" particles of reovirus were not generated, however "fragile" reoviral particles having very interesting characteristics were obtained after sonication and urea treatment. These treated particles were not able to replicate synthetic reoviral RNA, but current information obtained from these studies should be applicable to achieving in vitro replication. That could be accomplished by modification of conditions used to obtain "fragile" reoviral particle. Conditions were established in which soluble viral polymerase could be isolated.

Fig. 26. Electron micrographs of cores treated with 4.8 M urea and sonicated dialyzed cores following CsCl gradient centrifugation, both before and after subsequent sonication; (magnification x 190 000), (A) purified 4.8 M urea treated cores; (B) purified dialyzed cores sonicated for 12 min., (C) 4.8 M urea/1 min. sonicated cores; (D) dialyzed cores sonicated for 12 min. after additional sonication. Bar 100 nm.


A
B
D

5. DISCUSSION

5.1 Introduction of a synthetic M1 gene into reovirus

In earlier experiments by others, when reoviral protein/mRNA and helper virus were supplied, transfection of sets of 10 viral mRNAs led to rescue into viable virus (147). Reoviruses with 11 segments were obtained but were unstable (106). My attempts to introduce a synthetic M1 gene into reovirus by transfection of reovirus infected cells were unsuccessful. Experiments in which the fate of a transfected synthetic gene in reovirus infected cells was examined, showed that the transfected gene was not associated with the reoviral multiplication machinery. It was shown that the RNA is stable within the cell after transfection, that it probably undergoes translation, but no proof of association with any form of viral structure for any length of time was obtained. Alternatively the process of assembly of synthetic RNA might have taken place but with such low frequency that it was not detected with the techniques used. The synthetic mRNA, even if assembled, might not be able to propagate and thus would not be detected on subsequent passages of the virus. The reasons for failure to introduce a synthetic gene into reovirus might be as follows:

(i) Incorrect timing of transfection relative to the events of reoviral multiplication in infected cells.

(ii) Incorrect location of transfected RNA in the host cytoplasm. Reoviral ssRNAs are known to be assembled with reoviral proteins into particles in "viral factories" (88, 89). The requirements for entry of ssRNA into "factories" and assembly there, are not known.

(iii) Since viral mRNA (147) but not synthetic mRNA can be encapsidated into reoviral particles following transfection it is proposed that viral mRNA has some, as yet unidentified, modifications necessary for encapsidation.

(iv) It is possible that additional mutations in the synthetic M1D1 gene of type 3 reovirus will be required in order for it to be rescued into T1 reovirus. It was shown before that certain mutations in the reovirus genome are necessary for reassortment to occur. To be accepted by T1 reovirus the gene from different serotype has to have a mutation which renders it more similar to the T1 genome segment it replaces (106). The specific mutation introduced into synthetic T3 gene could permit its rescue by T1 reovirus.

(v) It is possible that since T1 reovirus does not generate deletion mutants as easily as T3 reovirus, it is not a good candidate as an acceptor for the delered genes. G2 reassortant reovirus might have been a better candidate for this experiment since it is known to generate deletions in the M1 gene. However this reassortant contains the T1M1 genome segment and can probably be considered to act as T1 virus (only S1, L2 and M3 of T3 origin) (105) so the constraints (mentioned in (iv)) on incorporation of M1T3 by heterologous virus would still hold. If T3 reovirus was to be used to rescue a synthetic T3M1 gene, then the transfected RNA would have to contain a marker in order to distinguish the synthetic gene from naturally generated M1 mutants.

(vi) It was observed for ϕ6 bacteriophage (139) that additional segments can be accepted by the virus if others are deleted. If that phenomenon also exists for reovirus then deletion mutants would be better acceptors of synthetic genes than wild type virus.

5.2 Generation of a lethal M1 mutant

It was assumed that on high multiplicity passage a wide array of mutants would be generated including point mutants and deletion mutants (Appendix II). UV treatment was used to increase the pool of mutants. UV irradiation is known to generate point mutations and in the case of reovirus to abolish its infectious potential. Indeed a decrease in titer was observed following passages and UV irradiation indicating that mutants were generated.

It was expected that M1 mutant particles would need the expression of μ2 protein in host cells, not for transcription and replication (since the mutant will carry wild type μ2 protein) but for production of viable progeny virus. This assumption was based on previous studies (37, 162) that showed that deleted M1 genes are transcribed, replicated by mutant particles and assembled, probably with the aid of the μ2 protein of helper virus, and that the temperature sensitive reovirus mutant (tsH 11.2), which defect was mapped to M1 gene, can be complemented by μ2 expression.

However, the selection of a M1 mutant by passage in μ2 expressing cells turned out to be very difficult. One possible candidate M1 mutant was selected but it was unstable and was lost during storage. The reasons for the difficulty in selecting M1 mutants in μ2 are obscure since M1 deletion mutants are easily obtained by serial undiluted passage (37), and a similar system was successfully used to select neuraminidase-minus mutants of influenza virus (165). The possible explanations are as follows:

(i) Ability of μ2 expression to complement tsH 11.2 M1 mutant indicates that point mutants might be easier to complement than deletion mutants. Undiluted passage is known to generate deletion mutants and point mutants. Point mutants were also certainly generated by UV treatment but it is possible that multiple mutations in other segments prevented successful complementation by μ2 expression.

(ii) The low level of μ2 expression in the host cells (5% of the level of infected cells (162)), might not be high enough for selection of M1 mutants generated by undiluted passage. These mutants might be lacking functions that cannot be complemented by the low level of μ2 protein expression, whereas tsH 11.2, which was complemented by this level of μ2, is partially active at the non permissive temperature. It can be assumed that more μ2 protein (approaching the level seen during infection) might be required to complement some μ2 function(s).

(iii) If the M1 mutants complemented by μ2 expression still need helper virus for multiplication (eg. to have a higher level of μ2 expression) then it may not be possible to obtain them as individual plaques.

(iv) The serial passage and UV irradiation may result in the generation of dominant negative mutants that produce noninfectious particles and cannot be complemented (166).

The lower titer of all reovirus serotypes on all types of μ2 expressing cells relative to normal control cell lines is an interesting observation. It is consistent with the results of Zou and Brown (162) which demonstrated a inhibitory function of M1 mRNA or its protein product (μ2) on the host, which in turn affects viral multiplication. Alternatively it is possible

that μ2 is inhibitory to reoviral multiplication itself. The effect of T1 and T3 μ2 expression on T1, T3 and G2 reovirus are worth further investigation.

5.3 Studies of reovirus particle structure and transcription

The difference in the structure of rotavirus and reovirus particles did not allow for the direct application of the experiment of Ramig (90) in which rotavirus "open"cores were able to replicate exogenous mRNA. Since dialysis in low ionic strength buffer, which disrupted or "opened" rotavirus core particles, did not alter reovirus structure, other chemical and physical treatments were imposed on the virion and core particles in order to "open" them. This was not accomplished with any of the treatments.

Three techniques were used to characterize chemically and physically treated particles; EM, RNase A digestion and transcriptase assay, and some indications of structural changes as a result of these treatments were obtained. With the exception of fraction #6 from CsCl purification of 4.8 M urea treated cores, the presence of particles, resistance to RNase A digestion and transcriptional activity always occurred together. When particles were disrupted, dsRNA was susceptible to RNase A and transcriptional activity was abolished.

Fraction #6 from CsCl purification of 4.8 M urea treated cores was found to contain a transcriptase complex that was not associated with the core particle. The fact that the dsRNA from fraction #6 was susceptible to RNase A and that the presence of particles was not detected by EM suggested complete destruction of particles. However, this fraction was transcriptionally active. The λ3 reoviral protein was detected in fraction #6 by silver staining, confirming its role in transcription. This preparation represents the first demonstration of a subcore component with transcriptase activity. The results of EM, RNase A treatment, silver

staining and the transcriptional assay for fraction #6 demonstrated that reoviral transcriptase does not have to be associated with viral particles in order to be functional.

Since no traces of viral proteins were found in other than #2 and #6 fractions of the CsCl gradient after purification of 4.8 M urea treated cores, it seems that the dsRNA and λ3 found in fraction #6 leaked from the core particle (fraction #2) rather than being generated by core disruption. It remains to be established :

(i) How to optimize the production of this transcriptase preparation. In our experiments it was detected following three treatments : 4.8 M urea followed by low ionic strength dialysis and high salt CsCl fractionation. However, it is possible that any one of these treatments or modification thereof could produce soluble active polymerase.

(ii) Whether the soluble polymerase preparation is able to perform more than one cycle of transcription. It is possible that the location of the transcriptase within the core structure is required for it to complete more than one cycle.

(iii) Whether the dsRNA and λ3 are associated in a complex or independently migrated to the same fraction in the CsCl gradient. It is known that λ1 and σ2 are able to bind reoviral dsRNA (22, 23), however no data are available for λ3.

This transcriptase preparation needs to be further characterized and may provide important information on the components of reovirus transcriptase complexes.

Experiments were undertaken in order to replicate and introduce synthetic full length and deleted (+) sense M1 ssRNA into variously treated reovirus. All attempts were unsuccessful. Cores treated with 4.8 M urea had minimally active replicase in one experiment

but only native ssRNA was replicated (preliminary finding). Replicase from fraction #6 might be able to replicate synthetic ssRNA since the transcriptase is particle-free and thus will be equally accessible to native and synthetic genes.

Attempts to control reovirus disassembly showed the difficulty of obtaining particles at an intermediate stage between the intact and completely disrupted particles. Our aim was to obtain very fragile particles so that their dsRNA would be RNase A susceptible. Six cycles of freezing and thawing of dialyzed cores resulted in particles with altered structure, as seen by EM, and very low transcriptional activity, which together might indicate partial disruption of those particles. Also additional sonication of CsCl purified dialyzed cores sonicated for 12 min. as well as purified cores treated with 4.8 M urea resulted in particles undergoing degradation (monitored by EM) and characterized by loss of, or very low transcriptional activity respectively. However the RNA in these preparations was not RNase A susceptible, which may have reflected the clumped nature of dsRNA which appears as intertwined strands after sonication.

Different chemical and physical treatments of reovirus resulted in particles with altered transcriptional activity. Reovirus transcription could be activated above the native core level. Higher reoviral transcription activity at both 34°C and 50°C was shown for heat treated cores heat treated dialyzed cores, and for pH 10.5 treated cores. Cores treated with pH 10.5 were not dialyzed prior to the transcription assay and their high transcriptional activity may only reflect a more optimal pH for transcriptase since the transcription assay was performed below the pH optimum established for reovirus transcriptase. The higher transcriptional activity of cores and dialyzed cores treated with heat was probably caused by structural changes that

allowed for faster elongation of the nascent chain. Elongation of transcripts was found to be the rate-limiting step in transcription of reovirus (167). Sonication of dialyzed cores, urea treatment (up to 4.8M) of cores, heat treatment of virus and 6 cycles of freeze/thaw of dialyzed cores resulted in particles that were more efficient in transcription at higher temperatures. On the other hand cores treated with 20% formamide and 10% 2ME had a lower temperature optimum than native cores.

It was shown previously (118) that prolonged storage of purified reovirus in phosphate buffer of low ionic strength results in the degradation of the reovirus capsid into subunits. This phenomenon was not observed with Tris buffer. Some treatments that resulted in complete (50% formamide) or partial (additional sonication of dialyzed cores sonicate for 12 min. and cores treated with 4.8 M urea) degradation of particles showed the presence of fibrous material and strands that probably represented reoviral genome dsRNA. The presence of similar structures was detected previously by EM after 5 min. of 0.01% SDS treatment, followed by 10 min. of high salt buffer (0.2 M) (8).

Lastly it was shown that virions activated by heat were able to produce full length reoviral ssRNA of all segments. It was shown previously that viral transcriptase can generate only short abortive transcripts within virions (65) and that heat activation of virion results in transcription of full lenght mRNAs within virions (67) which suggests that heat activation changes the structure of virion particle so that elongation of the nascent ssRNA is possible. The protrusions observed on the virus particle after heat activation may represent dsRNA leaving particles or some structurally altered reoviral proteins.

6. CONCLUSIONS

- Synthetic transcripts of reovirus M1 gene can be introduced into infected cells and appear to behave as reoviral M1 ssRNA but are not replicated or assembled into infectious virions.

- Lethal M1 mutants were not obtained by growth and plaque purification of mutated reovirus in cells that constitutively express μ2 protein.

- Although treatments of virion and core particles did not resulted in transcriptionally or replicationally active "open" particles, 4.8 M urea treatment resulted in release of viral transcriptase from reovirus particles. A fraction containing soluble, active transcriptase that consists of dsRNA and λ3 can be purified by CsCl centrifugation.

- Examination of physically and chemically treated cores and virions by RNase A, EM, and transcriptional assay demonstrated a relationship between particle integrity and transcriptional/replicational activity of reovirus. These studies might finally allow the construction of a reovirus rescue system.

7. REFERENCES

1. Joklik, W.K. The members of the family Reoviridae. The Reoviridae (Joklik W.K., Editor) Plenum Press, New York and London :1-6. 1983;

2. Loh, P.C. and Shatkin, A.J. Structural proteins of reoviruses. Journal of Virology 2:1353-9. 1968;

3. Smith, R.E., Zweerink, H.J., and Joklik, W.K. Polypeptide components of virions, top component and cores of reovirus type 3. Virology 39:791-810. 1969;

4. Both, G.W., Lavi, S., and Shatkin, A.J. Synthesis of all the gene products of the reovirus genome in vivo and in vitro. Cell 4:173-80. 1975;

5. McCrae, M.A. and Joklik, W.K. The nature of the polypeptide encoded by each of the 10 double-stranded RNA segments of reovirus type 3. Virology 89:578-93. 1978;

6. Cashdollar, L.W., Chmelo, R.A., Wiener, J.R., and Joklik, W.K. Sequences of the S1 genes of the three serotypes of reovirus. Proceedings of the National Academy of Sciences of the United States of America 82:24-8. 1985;

7. Lee, P.W., Hayes, E.C., and Joklik, W.K. Characterization of anti-reovirus immunoglobulins secreted by cloned hybridoma cell lines. Virology 108:134-46. 1981;

8. Luftig, R.B., Kilham, S.S., Hay, A.J., Zweerink, H.J., and Joklik, W.K. An ultrastructural study of virions and cores of reovirus type 3. Virology 48:170-81. 1972;

9. Leone, G., Maybaum, L., and Lee, P.W. The reovirus cell attachment protein possesses two independently active trimerization domains: basis of dominant negative effects. Cell 71:479-88. 1992;

10. Fraser, R.D., Furlong, D.B., Trus, B.L., Nibert, M.L., Fields, B.N., and Steven, A.C. Molecular structure of the cell-attachment protein of reovirus: correlation of computer-processed electron micrographs with sequence-based predictions. Journal of Virology 64:2990-3000. 1990;

11. Furlong, D.B., Nibert, M.L., and Fields, B.N. Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles. Journal of Virology 62:246-56. 1988;

12. Duncan, R. and Lee, P.W. Localization of two protease-sensitive regions separating distinct domains in the reovirus cell-attachment protein sigma 1. Virology 203:149-52. 1994;

13. Virgin, H.W., 4th, Mann, M.A., Fields, B.N., and Tyler, K.L. Monoclonal antibodies to reovirus reveal structure/function relationships between capsid proteins and genetics of susceptibility to antibody action. Journal of Virology 65:6772-81. 1991;

14. Larson, S.M., Antczak, J.B., and Joklik, W.K. Reovirus exists in the form of 13 particle species that differ in their content of protein sigma 1. Virology 201:303-11. 1994;

15. Dryden, K.A., Wang, G., Yeager, M., Nibert, M.L., Coombs, K.M., Furlong, D.B., Fields, B.N., and Baker, T.S. Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. Journal of Cell Biology 122:1023-41. 1993;

16. Nibert, M.L., Schiff, L.A., and Fields, B.N. Mammalian reoviruses contain a myristoylated structural protein. Journal of Virology 65:1960-7. 1991;

17. Nibert, M.L. and Fields, B.N. A carboxy-terminal fragment of protein mu 1/mu 1C is present in infectious subvirion particles of mammalian reoviruses and is proposed to have a role in penetration. Journal of Virology 66:6408-18. 1992;

18. Wessner, D.R. and Fields, B.N. Isolation and genetic characterization of ethanol-resistant reovirus mutants. Journal of Virology 67:2442-7. 1993;

19. Tillotson, L. and Shatkin, A.J. Reovirus polypeptide sigma 3 and N-terminal myristoylation of polypeptide mu 1 are required for site-specific cleavage to mu 1C in transfected cells. Journal of Virology 66:2180-6. 1992;

20. Mabrouk, T. and Lemay, G. The sequence similarity of reovirus sigma 3 protein to picornaviral proteases is unrelated to its role in mu 1 viral protein cleavage. Virology 202:615-20. 1994;

21. White, C.K. and Zweerink, H.J. Studies on the structure of reovirus cores: selective removal of polypeptide lambda 2. Virology 70:171-80. 1976;

22. Schiff, L.A., Nibert, M.L., Co, M.S., Brown, E.G., and Fields, B.N. Distinct binding sites for zinc and double-stranded RNA in the reovirus outer capsid protein sigma 3. Molecular & Cellular Biology 8:273-83. 1988;

23. Lemay, G. and Danis, C. Reovirus lambda 1 protein: affinity for double-stranded nucleic acids by a small amino-terminal region of the protein independent from the zinc finger motif. Journal of General Virology 75:3261-6. 1994;

24. Harvey, J.D., Bellamy, A.R., Earnshaw, W.C., and Schutt, C. Biophysical studies of reovirus type 3. IV. Low-angle x-ray diffraction studies. Virology 112:240-9. 1981;

25. Lepault, J., Dubochet, J., Baschong, W., and Kellenberger, E. Organization of double-stranded DNA in bacteriophages: a study by cryo-electron microscopy of vitrified samples. EMBO Journal 6:1507-12. 1987;

26. Vasquez, C. and Kleinschmidt, A.K. Electron microscopy of RNA strands released from individual Reovirus particles. Journal of Molecular Biology 34:137-47. 1968;

27. Kavenoff, R., Talcove, D., and Mudd, J.A. Genome-sized RNA from reovirus particles. Proceedings of the National Academy of Sciences of the United States of America 72:4317-21. 1975;

28. Shatkin, A.J. and Sipe, J.D. Single-stranded, adenine-rich RNA from purified reoviruses. Proceedings of the National Academy of Sciences of the United States of America 59:246-53. 1968;

29. Zarbl, H, Hastings, K.E.M., Millward, S. Reovirus core particles synthesize capped oligonucleotides as a result of abortive transcription. Archives of Biochemistry and Biophysics 202:348-60. 1980;

30. Coombs, K.M., Fields, B.N., and Harrison, S.C. Crystallization of the reovirus type 3 Dearing core. Crystal packing is determined by the lambda 2 protein. Journal of Molecular Biology 215:1-5. 1990;

31. Joklik, W.K. The members of the family Reoviridae. The Reoviridae (Joklik W.K., Editor) Plenum Press, New York and London :9-70. 1983;

32. Schiff, L.A. and Fields, B.N. Reoviruses and their replication. Virology.(Fields, B.N., Knipe, D.M., et al editors) New York: Raven Press Ltd. P.1275-306. 1990;

33. Wiener, J.R. and Joklik, W.K. The sequences of the reovirus serotype 1, 2, and 3 L1 genome segments and analysis of the mode of divergence of the reovirus serotypes. Virology 169:194-203. 1989;

34. Wiener, J.R., Bartlett, J.A., and Joklik, W.K. The sequences of reovirus serotype 3 genome segments M1 and M3 encoding the minor protein mu 2 and the major nonstructural protein mu NS, respectively. Virology 169:293-304. 1989;

35. Antczak, J.B., Chmelo, R., Pickup, D.J., and Joklik, W.K. Sequence at both termini of the 10 genes of reovirus serotype 3 (strain Dearing). Virology 121:307-19. 1982;

36. Chapell, J.D., Goral, M.I., Rodgers, S.E., dePamphilis, C.W., and Dermody, T.S. Sequence diversity within the reovirus S2 gene: reovirus genes reassort in nature, and their termini are predicted to form a panhandle motif. Journal of Virology 68:750-6. 1994;

37. Zou, S. and Brown, E.G. Identification of sequence elements containing signals for replication and encapsidation of the reovirus M1 genome segment. Virology 186:377-88. 1992;

38. Zou, S. and Brown, E.G. Nucleotide sequence comparison of the M1 genome segment of reovirus type 1 Lang and type 3 Dearing. Virus Research 22:159-64. 1992;

39. Banerjee, A.K. and Shatkin, A.J. Guanosine-5'-diphosphate at the 5' termini of reovirus RNA: evidence for a segmented genome within the virion. Journal of Molecular Biology 61:643-53. 1971;

40. Shatkin, A.J. Methylated messenger RNA synthesis in vitro by purified reovirus. Proceedings of the National Academy of Sciences of the United States of America 71:3204-7. 1974;

41. Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A.J. Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m-7G(5')ppp(5')G-MpCp-. Proceedings of the National Academy of Sciences of the United States of America 72:362-6. 1975;

42. Sauve, G.J., Saragovi, H.U., and Greene, M.I. Reovirus receptors. [Review]. Advances in Virus Research 42:325-41. 1993;

43. Strong, J.E., Tang, D., and Lee, P.W. Evidence that the epidermal growth factor receptor on host cells confers reovirus infection efficiency. Virology 197:405-11. 1993;

44. Tang, D., Strong, J.E., and Lee, P.W. Recognition of the epidermal growth factor receptor by reovirus. Virology 197:412-4. 1993;

45. Choi, A.H. Internalization of virus binding proteins during entry of reovirus into K562 erythroleukemia cells. Virology 200:301-6. 1994;

46. Lee, P.W., Hayes, E.C., and Joklik, W.K. Protein sigma 1 is the reovirus cell attachment protein. Virology 108:156-63. 1981;

47. Turner, D.L., Duncan, R., and Lee, P.W. Site-directed mutagenesis of the C-terminal portion of reovirus protein sigma 1: evidence for a conformation-dependent receptor binding domain. Virology 186:219-27. 1992;

48. Fernandes, J., Tang, D., Leone, G., and Lee, P.W. Binding of reovirus to receptor leads to conformational changes in viral capsid proteins that are reversible upon virus detachment. Journal of Biological Chemistry 269:17043-7. 1994;

49. Amerongen, H.M., Wilson, G.A., Fields, B.N., and Neutra, M.R. Proteolytic processing of reovirus is required for adherence to intestinal M cells. Journal of Virology 68:8428-32. 1994;

50. Tosteson, M.T., Nibert, M.L., and Fields, B.N. Ion channels induced in lipid bilayers by subvirion particles of the nonenveloped mammalian reoviruses. Proceedings of the National Academy of Sciences of the United States of America 90:10549-52. 1993;

51. Lucia-Jandris, P., Hooper, J.W., and Fields, B.N. Reovirus M2 gene is associated with chromium release from mouse L cells. Journal of Virology 67:5339-45. 1993;

52. Hazelton, P.R. and Coombs, K.M. The reovirus mutant tsA279 has temperature-sensitive lesions in the M2 and L2 genes: the M2 gene is associated with decreased viral protein production and blockade in transmembrane transport. Virology 207:46-58. 1995;

53. Shatkin, A.J. and Sipe, J.D. RNA polymerase activity in purified reoviruses. Proceedings of the National Academy of Sciences of the United States of America 61:1462-9. 1968;

54. Borsa, J. and Graham, A.F. Reovirus: RNA polymerase activity in purified virions. Biochemical & Biophysical Research Communications 33:895-901. 1968;

55. Silverstein, S.C., Christman, J.K., and Acs, G. The reovirus replicative cycle. Annual Review of Biochemistry 45:375-408. 1976;

56. Bartlett, N.M., Gillies, S.C., Bullivant, S., and Bellamy, A.R. Electron microscopy study of reovirus reaction cores. Journal of Virology 14:315-26. 1974;

57. Cashdollar, L.W. Characterization and structural localization of the reovirus lambda 3 protein. Research in Virology 145:277-85. 1994;

58. Morozov, S.Y. A possible relationship of reovirus putative RNA polymerase to polymerases of positive-strand RNA viruses. Nucleic Acids Research 17:5394 1989;

59. Bruenn, J.A. Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. Nucleic Acids Research 19:217-26. 1991;

60. Drayna, D. and Fields, B.N. Activation and characterization of the reovirus transcriptase: genetic analysis. Journal of Virology 41:110-8. 1982;

61. Starnes, M.C. and Joklik, W.K. Reovirus protein lambda 3 is a poly(C)-dependent poly(G) polymerase. Virology 193:356-66. 1993;

62. Yin, P. and Coombs, K.M. The M1 gene is associated with differences in the temperature optimum of the transcriptase activity in reovirus core particles.[In Press];

63. Mao, Z.X. and Joklik, W.K. Isolation and enzymatic characterization of protein lambda 2, the reovirus guanylyltransferase. Virology 185:377-86. 1991;

64. Powell, K.F., Harvey, J.D., and Bellamy, A.R. Reovirus RNA transcriptase: evidence for a conformational change during activation of the core particle. Virology 137:1-8. 1984;

65. Yamakawa, M., Furuichi, Y., and Shatkin, A.J. Reovirus transcriptase and capping enzymes are active in intact virions. Virology 118:157-68. 1982;

66. Skehel, J.J. and Joklik, W.K. Studies on the in vitro transcription of reovirus RNA catalyzed by reovirus cores. Virology 39:822-31. 1969;

67. Banerjee, A.K. and Shatkin, A.J. Transcription in vitro by reovirus-associated ribonucleic acid-dependent polymerase. Journal of Virology 6:1-11. 1970;

68. Bellamy, A.R. and Harvey, J.D. Biophysical studies of reovirus type 3. III. A laser light-scattering study of the RNA transcriptase reaction. Virology 70:28-36. 1976;

69. Lau, R.Y., Van Alstyne, D., Berckmans, R., and Graham A.F. Synthesis of reovirus-specific polypeptides in cells pretreated with cycloheximide. Journal of Virology 16:470-8. 1975;

70. Spandidos, D.A., Krystal, G., and Graham, A.F. Regulated transcription of the genomes of defective virions and temperature-sensitive mutants of reovirus. Journal of Virology 18:7-19. 1976;

71. Zweerink, H.J. and Joklik, W.K. Studies on the intracellular synthesis of reovirus-specified proteins. Virology 41:501-18. 1970;

72. Roner, M.R., Gaillard, R.K., Jr., and Joklik, W.K. Control of reovirus messenger RNA translation efficiency by the regions upstream of initiation codons. Virology 168:292-301. 1989;

73. Kozak, M. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Research 9:5233-62. 1981;

74. Belli, B.A. and Samuel, C.E. Biosynthesis of reovirus-specified polypeptides: identification of regions of the bicistronic reovirus S1 mRNA that affect the efficiency of translation in animal cells. Virology 193:16-27. 1993;

75. Doohan, J.P. and Samuel, C.E. Biosynthesis of reovirus-specified polypeptides:Ribosome pausing during translation of reovirus S1 mRNA. Virology 186:409-25. 1992;

76. Roner, M.R., Roner, L.A., and Joklik, W.K. Translation of reovirus RNA species m1 can initiate at either of the first two in-frame initiation codons. Proceedings of the National Academy of Sciences of the United States of America 90:8947-51. 1993;

77. Zou, S. and Brown, E.G. Translation of the reovirus M1 gene initiates from the first AUG codon in both infected and transfected cells.[In Press];

78. Zarbl, H., Skup, D., and Millward, S. Reovirus progeny subviral particles synthesize uncapped mRNA. Journal of Virology 34:497-505. 1980;

79. Chang, C.T. and Zweerink, H.J. Fate of parental reovirus in infected cell. Virology 46:544-55. 1971;

80. Fajardo, E. and Shatkin, A.J. Expression of the two reovirus S1 gene products in transfected mammalian cells. Virology 178:223-31. 1990;

81. Belli, B.A. and Samuel, C.E. Biosynthesis of reovirus-specific peptides: Expression of reovirus S1-encoded sigma 1NS protein in transfected and infected cells as measured with serotype specific polyclonal antibody. Virology 185:698-709. 1991;

82. Tyler, K.L., Squier, M.K., Rodgers, S.E, Schneider, B.E., Oberhaus, S.M., Grdina, T.A., Cohen, J.J., and Dermody, T.S. Difference in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein sigma 1. Journal of Virology 69:6972-9. 1995;

83. Lloyd, R.M. and Shatkin, A.J. Translational stimulation by reovirus polypeptide sigma 3: substitution for VAI RNA and inhibition of phosphorylation of the alpha subunit of eukaryotic initiation factor 2. Journal of Virology 66:6878-84. 1992;

84. Beattie, E., Denzler, K.L., Tartaglia, J., Perkus, M.E., Paoletti, E., and Jacobs, B.L. Reversal of the interferon-sensitive phenotype of a vaccinia virus lacking E3L by expression of the reovirus S4 gene. Journal of Virology 69:499-505. 1995;

85. Martin, P.E. and McCrae, M.A. Analysis of the stimulation of reporter gene expression by the sigma 3 protein of reovirus in co-transfected cells. Journal of General Virology 74:1055-62. 1993;

86. Seliger, L.S., Giantini, M., and Shatkin, A.J. Translational effects and sequence comparisons of the three serotypes of the reovirus S4 gene. Virology 187:202-10. 1992;

87. Sharpe, A.H. and Fields, B.N. Reovirus inhibition of cellular RNA and protein synthesis: role of the S4 gene. Virology 122:381-91. 1982;

88. Sharpe, A.H., Chen, L.B., and Fields, B.N. The interaction of mammalian reoviruses with the cytoskeleton of monkey kidney CV-1 cells. Virology 120:399-411. 1982;

89. Mora, M., Partin, K., Bhatia, M., Partin, J., and Carter, C. Association of reovirus proteins with the structural matrix of infected cells. Virology 159:265-77. 1987;

90. Antczak, J.B. and Joklik, W.K. Reovirus genome segment assortment into progeny genomes studied by the use of monoclonal antibodies directed against reovirus proteins. Virology 187:760-76. 1992;

91. Huismans, H. and Joklik, W.K. Reovirus-coded polypeptides in infected cells: isolation of two native monomeric polypeptides with affinity for single-stranded and double-stranded RNA, respectively. Virology 70:411-24. 1976;

92. Miller, J.E. and Samuel, C.E. Proteolytic cleavage of the reovirus sigma 3 protein results in enhanced double-stranded RNA-binding activity: identification of a repeated basic amino acid motif within the C-terminal binding region. Journal of Virology 66:5347-56. 1992;

93. Denzler, K.L. and Jacobs, B.L. Site-directed mutagenic analysis of reovirus sigma 3 protein binding to dsRNA. Virology 204:190-9. 1994;

94. Mabrouk, T. and Lemay, G. Mutations in a CCHC zinc-binding motif of the reovirus sigma 3 protein decrease its intracellular stability. Journal of Virology 68:5287-90. 1994;

95. Kedl, R., Schmechel, S., and Schiff, L. Comparative sequence analysis of the reovirus S4 genes from 13 serotype 1 and serotype 3 field isolates. Journal of Virology 69:552-9. 1995;

96. Ito, Y. and Joklik, W.K. Temperature-sensitive mutants of reovirus. II. Anomalous electrophoretic migration of certain hybrid RNA molecules composed of mutant plus strands and wild-type minus strands. Virology 50:202-8. 1972;

97. Ramig, R.F., Mustoe, T.A., Sharpe, A.H., and Fields, B.N. A genetic map of reovirus II. Assignment of the double-stranded RNA - negative mutant groups C, D and E to genome segments. Virology 85:531-44. 1978;

98. Coombs, K.M., Mak, S.C., and Petrycky-Cox, L.D. Studies of the major reovirus core protein sigma 2: reversion of the assembly-defective mutant tsC447 is an intragenic process and involves back mutation of Asp-383 to Asn. Journal of Virology 68:177-86. 1994;

99. Sakuma, S. and Watanabe, Y. Unilateral synthesis of reovirus double-stranded ribonucleic acid by a cell-free replicase system. Journal of Virology 8:190-6. 1971;

100. Morgan, E.M. and Zweerink, H.J. Characterization of transcriptase and replicase particles isolated from reovirus-infected cells. Virology 68:455-66. 1975;

101. Zweerink, H.J., Morgan, E.M., and Skyler, J.S. Reovirus morphogenesis: characterization of subviral particles in infected cells. Virology 73:442-53. 1976;

102. Ramig, R.F. Principles of animal virus genetics. Virology.(Fields, B.N., Knipe, D.M., et al editors) New York: Raven Press Ltd. p. 95-122. 1990;

103. Nonoyama, M., Watanabe, Y., and Graham, A.F. Defective virions of reovirus. Journal of Virology 6:226-36. 1970;

104. Nonoyama, M. and Graham, A.F. Appearance of defective virions in clones of reovirus. Journal of Virology 6:693-4. 1970;

105. Brown, E.G., Nibert, M.L., Fields, B.N. The L2 gene of reovirus serotype 3 controls the capacity to interfere, accumulate deletions and establish persistent infections. Double-Stranded RNA Viruses. (Compans, R.W., Bishop, D.H.L. editors) Amsterdam: Elsevier Science Publishing Co., Inc. p. 275-87. 1983;

106. Joklik, W.K. and Roner, M.R. What reassorts when reovirus genome segments reassort?. [Review]. Journal of Biological Chemistry 270:4181-4. 1995;

107. Selb, B. and Weber, B. A study of human reovirus IgG and IgA antibodies by ELISA and Western blot. Journal of Virological Methods 47:15-26. 1994;

108. Tyler, K.L., and Fields, B.N. Reoviruses. Virology. (Fields, B.N., Knipe, D.M., et al editors) New York: Raven Press Ltd. p. 1307-28. 1990;

109. Wilson, G.A., Morrison, L.A., and Fields, B.N. Association of the reovirus S1 gene with serotype 3-induced biliary atresia in mice. Journal of Virology 68:6458-65. 1994;

110. Rozinov, M.N. and Fields, B.N. Interference following mixed infection of reovirus isolates is linked to the M2 gene. Journal of Virology 68:6667-71. 1994;

111. Sherry, B. and Blum, M.A. Multiple viral core proteins are determinants of reovirus-induced acute myocarditis. Journal of Virology 68:8461-5. 1994;

112. Haller, B.L., Barkon, M.L., Vogler, G.P., and Virgin, H.W., 4th. Genetic mapping of reovirus virulence and organ tropism in severe combined immunodeficient mice: organ-specific virulence genes. Journal of Virology 69:357-64. 1995;

113. Matoba, Y., Colucci, W.S., Fields, B.N., and Smith, T.W. The reovirus M1 gene determines the relative capacity of growth of reovirus in cultured bovine aortic endothelial cells. Journal of Clinical Investigation 92:2883-8. 1993;

114. Matoba, Y., Sherry, B., Fields, B.N., and Smith, T.W. Identification of the viral genes responsible for growth of strains of reovirus in cultured mouse heart cells. Journal of Clinical Investigation 87:1628-33. 1991;

115. Hayes, E.C., Lee, P.W., Miller, S.E., and Joklik, W.K. The interaction of a series of hybridoma IgGs with reovirus particles. Demonstration that the core protein lambda 2 is exposed on the particle surface. Virology 108:147-55. 1981;

116. Virgin, H.W., 4th, Mann, M.A., and Tyler, K.L. Protective antibodies inhibit reovirus internalization and uncoating by intracellular proteases. Journal of Virology 68:6719-29. 1994;

117. Tyler, K.L., Mann, M.A., Fields, B.N., and Virgin, H.W., 4th. Protective anti-reovirus monoclonal antibodies and their effects on viral pathogenesis. Journal of Virology 67:3446-53. 1993;

118. Amano, Y., Katagiri, S., Ishida, N., and Watanabe, Y. Spontaneous degradation of reovirus capsid into subunits. Journal of Virology 8:805-8. 1971;

119. Drayna, D. and Fields, B.N. Biochemical studies on the mechanism of chemical and physical inactivation of reovirus. Journal of General Virology 63:161-70. 1982;

120. Almeida, J.D., Bradburne, A.F., and Wreghitt, T.G. The effect of sodium thiocyanate on virus structure. Journal of Medical Virology 4:269-77. 1979;

121. Levin, D.H., Mendelsohn, N., Schonberg, M., Klett, H., Silverstein, S., Kapuler, A.M., and Acs, G. Properties of RNA transcriptase in reovirus subviral particles. Proceedings of the National Academy of Sciences of the United States of America 66:890-7. 1970;

122. Moss, B. Vaccinia virus: a tool for research and vaccine development. [Review]. Science 252:1662-7. 1991;

123. Luytjes, W., Krystal, M., Enami, M., Pavin, J.D., and Palese, P. Amplification, expression, and packaging of foreign gene by influenza virus. Cell 59:1107-13. 1989;

124. Enami, M., Luytjes, W., Krystal, M., and Palese, P. Introduction of site-specific mutations into the genome of influenza virus. Proceedings of the National Academy of Sciences of the United States of America 87:3802-5. 1990;

125. Percy, N., Barclay, W.S., Garcia-Sastre, A., and Palese, P. Expression of a foreign protein by influenza A virus. Journal of Virology 68:4486-92. 1994;

126. Garcia-Sastre, A., Muster, T., Barclay, W.S., Percy, N., and Palese, P. Use of a mammalian internal ribosomal entry site element for expression of a foreign protein by a transfectant influenza virus. Journal of Virology 68:6254-61. 1994;

127. Neumann, G., Zobel, A., and Hobom, G. RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202:477-9. 1994;

128. Collins, P.L., Mink, M.A., and Stec, D.S. Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. Proceedings of the National Academy of Sciences of the United States of America 88:9663-7. 1991;

129. Collins, P.L., Mink, M.A., Hill, M.G., 3d, Camargo, E., Grosfeld, H., and Stec, D.S. Rescue of a 7502-nucleotide (49.3% of full-length) synthetic analog of respiratory syncytial virus genomic RNA. Virology 195:252-6. 1993;

130. Sidhu, M.S., Chan, J., Kaelin, K., Spielhofer, P., Radecke, F., Schneider, H., Masurekar, M., Dowling, P.C., Billeter, M.A., and Udem, S.A. Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. Virology 208:800-7. 1995;

131. Park, K.H., Huang, T., Correia, F.F., and Krystal, M. Rescue of a foreign gene by Sendai virus. Proceedings of the National Academy of Sciences of the United States of America 88:5537-41. 1991;

132. Dimock, K. and Collins, P.L. Rescue of synthetic analogs of genomic RNA and replicative-intermediate RNA of human parainfluenza virus type 3. Journal of Virology 67:2772-8. 1993;

133. Pattnaik, A.K., Ball, L.A., LeGrone, A.W., and Wertz, G.W. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69:1011-20. 1992;

134. Conzelmann, K.K. and Schnell, M. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. Journal of Virology 68:713-9. 1994;

135. Fujimura, T. and Wickner, R.B. Replicase of L-A virus-like particles of Saccharomyces cerevisiae. In vitro conversion of exogenous L-A and M1 single-stranded RNAs to double-stranded form. Journal of Biological Chemistry 263:454-60. 1988;

136. Fujimura, T., Ribas, J.C., Makhov, A.M., and Wickner, R.B. Pol of gag-pol fusion protein required for encapsidation of viral RNA of yeast L-A virus. Nature 359:746-9. 1992;

137. Yao, W., Muqtadir, K., and Bruenn, J.A. Packaging in a yeast double-stranded RNA virus. Journal of Virology 69:1917-9. 1995;

138. Qiao, X., Casini, G., Qiao, J., and Mindich, L. In vitro packaging of individual genomic segments of bacteriophage phi 6 RNA: serial dependence relationships. Journal of Virology 69:2926-31. 1995;

139. Onodera, S., Qiao, X., Qiao, J., and Mindich, L. Acquisition of a fourth genomic segment in bacteriophage phi 6, a bacteriophage with a genome of three segments of dsRNA. Virology 212:204-12. 1995;

140. Olkkonen, V.M., Gottlieb, P., Strassman, J., Qiao, X.Y., Bamford, D.H., and Mindich, L. In vitro assembly of infectious nucleocapsids of bacteriophage phi 6: formation of a recombinant double-stranded RNA virus. Proceedings of the National Academy of Sciences of the United States of America 87:9173-7. 1990;

141. Onodera, S., Olkkonen, V.M., Gottlieb, P., Strassman, J., Qiao, X.Y., Bamford, D.H., and Mindich, L. Construction of a transducing virus from double-stranded RNA bacteriophage phi6: establishment of carrier states in host cells. Journal of Virology 66:190-6. 1992;

142. Xu, P., Miller, S.E., and Joklik, W.K. Generation of reovirus core-like particles in cells infected with hybrid vaccinia viruses that express genome segments L1, L2, L3, and S2. Virology 197:726-31. 1993;

143. Crawford, S.E., Labbe, M., Cohen, J., Burroughs, M.H., Zhou, Y.J., and Estes, M.K. Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. Journal of Virology 68:5945-22. 1994;

144. Loudon, P.T. and Roy, P. Assembly of five bluetongue virus proteins expressed by recombinant baculoviruses: inclusion of the largest protein VP1 in the core and virus-like proteins. Virology 180:798-802. 1991;

145. Chen, D., Zeng, C.Q., Wentz, M.J., Gorziglia, M., Estes, M.K., and Ramig, R.F. Template-dependent, in vitro replication of rotavirus RNA. Journal of Virology 68:7030-9. 1994;

146. Chen, D. and Ramig, R.F. Rescue of infectivity by sequential in vitro transcapsidation of rotavirus core particles with inner capsid and outer capsid proteins. Virology 194:743-51. 1993;

147. Roner, M.R., Sutphin, L.A., and Joklik, W.K. Reovirus RNA is infectious. Virology 179:845-52. 1990;

148. Montgomery, L.B., Kao, C.Y., Verdin, E., Cahill, C., and Maratos-Flier, E. Infection of a polarized epithelial cell line with wild-type reovirus leads to virus persistence and altered cellular function. Journal of General Virology 72:2939-46. 1991;

149. Dermody, T.S., Nibert, M.L., Wetzel, J.D., Tong, X., and Fields, B.N. Cells and viruses with mutations affecting viral entry are selected during persistent infections of L cells with mammalian reoviruses. Journal of Virology 67:2055-63. 1993;

150. Morrison, L.A., Fields, B.N., and Dermody, T.S. Prolonged replication in the mouse central nervous system of reoviruses isolated from persistently infected cell cultures. Journal of Virology 67:3019-26. 1993;

151. Dermody, T.S., Chappell, J.D., Hofler, J.G., Kramp, W., and Tyler, K.L. Eradication of persistent reovirus infection from a B-cell hybridoma. Virology 212:272-6. 1995;

152. Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular Cloning: A laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, p. 1.25-41. 1989;

153. Birnboim, H.C. and Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7:1513-23. 1979;

154. Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular Cloning: A laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, p. 1.85. 1989;

155. Sambrook, J., Fritsch, E.F., Maniatis, T. Molecular Cloning: A laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, p. 1.90-99. 1989;

156. Brown, E.G. Vesiculovirus: comparisons of protein structure and studies of the abnormal N protein of the vesicular stomatis New Jersey D1 temperature - sensitive mutant [Ph.D.Thesis]. p.60 1981;

157. Protocol and application guide. Promega, p. 43. 1989/90;

158. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielson, M. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences of the United States of America 64:7413-7. 1987;

159. Schmid, A., Cattaneo, R., and Billeter, M.A. A procedure for selective full length cDNA cloning of specific RNA species. Nucleic Acids Research 15:3987-96. 1987;

160. Schaffhausen, B.S., Silver, J.E., and Benjamin, T.L. Tumor antigen(s) in cell productively infected by wild-type polyoma virus and mutant NG-18. Proceedings of the National Academy of Sciences of the United States of America 75:79-83. 1978;

161. Brown, E.G. Vesiculovirus: comparisons of protein structure and studies of the abnormal N protein of the vesicular stomatis New Jersey D1 temperature - sensitive mutant [Ph.D.Thesis]. p.49-50 1981;

162. Zou, S., and Brown, E.G. Stable expression of the reovirus mu 2 protein in mouse L cells complements the growth of a reovirus ts mutant with a defect in its M1 gene. [In Press];

163. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5. 1970;

164. Merril, C.R., Goldman, D., Sedman, S.A., and Ebert, M.H. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211:1437-8. 1981;

165. Liu, C. and Air, G.M. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. Virology 194:403-7. 1993;

166. Whitaker-Dowling, P., Lucas, W., and Youngner, J.S. Cold-adapted vaccine strains of influenza A virus act as dominant negative mutants in mixed infections with wild-type influenza A virus. Virology 175:358-64. 1990;

167. Zarbl, H., and Millward, S. The reovirus multiplication cycle. The Reoviridae (Joklik, W.K. Editor) Plenum Press, New York and London :107-195. 1983;

8. APPENDIX I

LB Medium (Luria - Bertani Medium), pH 7.5

bacto - tryptone	1% (w/v)
bacto - yeast extract	0.5% (w/v)
NaCl	1% (w/v)

1.5% (w/v) agar added for LB plates

TB (Terrific Broth Medium)

bacto - tryptone	1.2% (w/v)
bacto - yeast extract	2.4% (w/v)
glycerol	0.4% (w/v)
KH_2PO_4	17 mM
K_2HPO_4	72 mM

2YT, pH 7.0

bacto - tryptone	1.6% (w/v)
bacto - yeast extract	1% (w/v)
NaCl	0.5% (w/v)

SOC

bacto - tryptone	2% (w/v)
bacto - yeast extract	0.5% (w/v)
NaCl	0.05% (w/v)
KCl	2.5 mM
$MgCl_2$	10 mM
glucose	20 mM

TE, pH 8.0

Tris-HCl	10 mM pH 8.0
EDTA	1 mM pH 8.0

PBS 1x

NaCl	145 mM
NaH_2PO_4	2.2 mM
Na_2HPO_4	7.6 mM

TBE 1x

Tris-borate	89 mM
EDTA	2mM pH 8

SSC 20x

NaCl 3M

sodium citrate 3M pH 7.0

Denhardt's 50x

Bovine serum albumine	1% (w/v)
Ficoll	1% (w/v)
Polyvinylpyrroline (pvp)	1% (w/v)
SDS	1% (w/v)

Primers

BogPriT3

5' TCA AGA GGA TGG CAT GCT CAC AGA T 3'

M15(-)(-G)

5' CTG CAG ATG AAG CGC GTA CGT AG 3'

SZ5+

5' GTA ATA CGA CTC ACT ATA GCT ATT CGC GGT CAT GGC 3'

SZ7-

5' TAT GAA TGC AGA TGA AGC GCG TAC GTA G 3'

SZ8-

5' GTA ATA CGA CTC ACT ATA GAT GAA GCG CGT ACG TAG 3'

SZ9+

5' TAT GAA TGC AGC TAT TCG CGG TCA TGG C 3'

M15'(-)

5' TAA CTG CAG GAT GAA GCG CGT ACG TAG 3'

M15'(+)

5' TAA CTG CAG GCT ATT CGC GGT CAT GGC 3'

9. APPENDIX II

9.1 Selection of lethally mutated reovirus

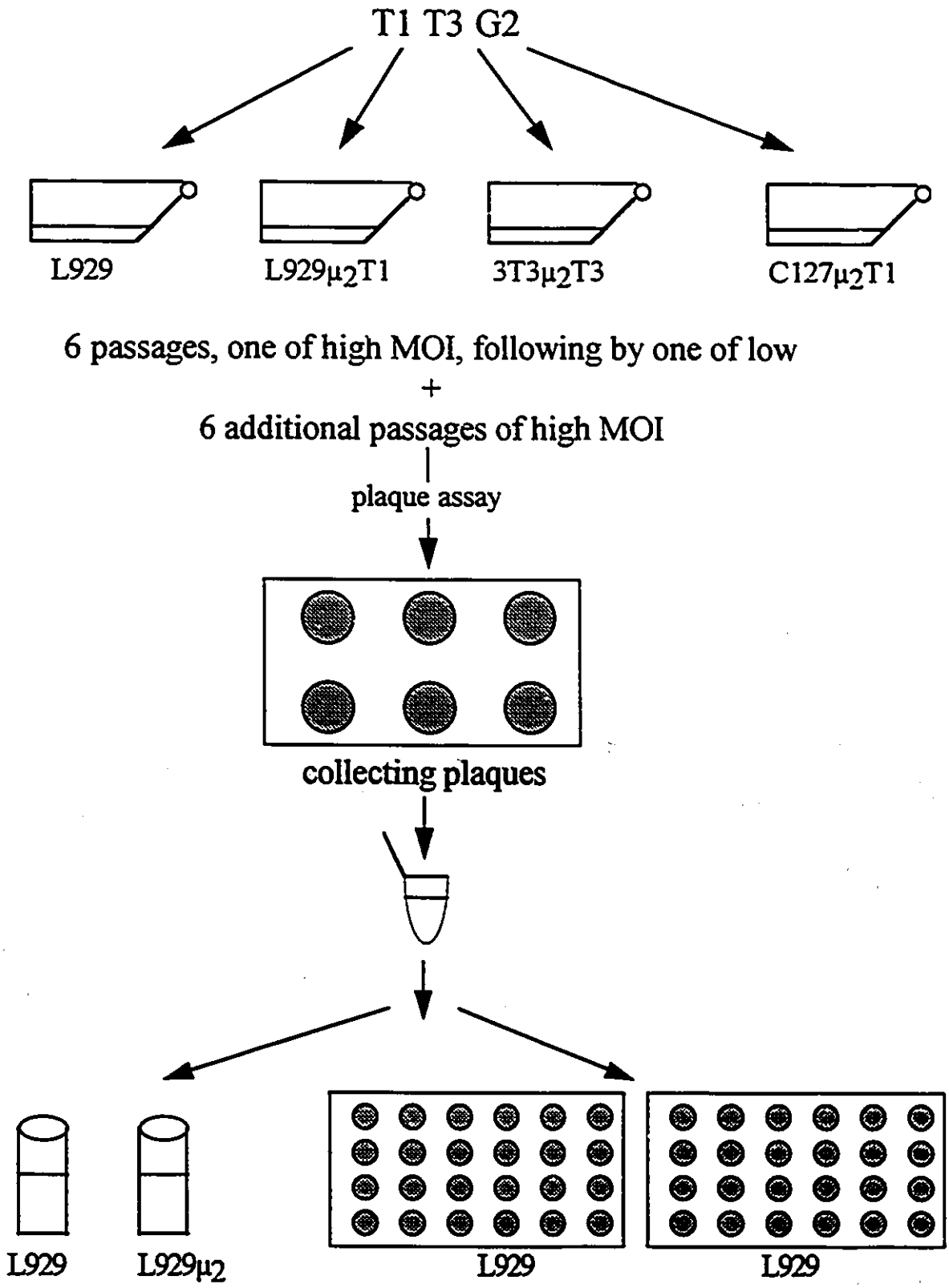
9.1.1 Selection of the M1 mutant from the virus population by serial undiluted passage in μ2 expressing cells

The generation of M1 reovirus mutants was performed by serial undiluted passage in μ2 expressing cells. Serial undiluted passage is known to generate stocks of defective virus (37, 105, 103, 104). It was expected that M1 mutants would be selected from the pool of other deletion mutants by passage in μ2 expressing cells. The mutants could then be used as acceptors of synthetic reoviral ssRNA. The mutants accepting the foreign gene would rescue the wild type phenotype and thus could be selected by growth on normal L929 cells.

Three types of reovirus: T1, T3 and G2 reovirus were passaged on 3 types of cells expressing μ2 protein. G2 reovirus - T1xT3 reassortant was particularly useful for this study due to the presence of the L2 segment from T3 and the M3 segment from T1, and thus its ability to generate deletions of M1 gene on serial undiluted passage (105).

Cells expressing μ2 protein were generated in our lab (162), and the ones used to select M1 mutants were: 3T3 cells expressing T3μ2 protein, L929 cell expressing T1μ2 protein and C127 expressing T1μ2 protein. Normal L929 cells were used as controls (Fig. 27).

Fig. 27. Schematic representation of the selection of the lethal M1 mutant by serial passage in μ2 expressing cells.


T1 T3 G2
L929
L929μ2T1
3T3μ2T3
C127μ2T1
6 passages, one of high MOI, following by one of low
+
6 additional passages of high MOI
plaque assay
collecting plaques
L929
L929μ2
L929
L929

The viruses were passaged 6 times, alternating diluted and undiluted passage followed by another 6 undiluted passage. Viruses from different passages were plaqued on μ2 expressing cells and normal L929 cells. An increase in plaque number on μ2 expressing cells was expected assuming that viruses passaged on μ2 expressing cells have selected M1 mutants due to complementation by the μ2 protein coexpressed by the cell. Such M1 mutants would lose the ability to multiply in the absence of μ2 protein. It was expected that on subsequent passage there would be fewer viruses that would be able to grow on normal L929 cells. However, the titer of virus was 1.06 - 6.6 fold higher on L929 cells than on μ2 expressing cells (Table 3).

Table 3. Analysis of T1, T3 and G2 titers following passage in normal L929 cells and μ2 expressing L929 and C127 cells

Cell Type/ Passage	L929 (pfu/ml)	L929μ2 (pfu/ml)	C127μ2 (pfu/ml)
T1P6/3T3μ2	$3.0x10^9$	$1.0x10^9$	
T3P6/3T3μ2	$5.5x10^8$	$1.4x10^8$	
G2P6/3T3μ2	$4.0x10^7$	$2.5x10^7$	
T1P8/3T3μ2	$2.0x10^8$	$1.9x10^8$	$1.2x10^8$
T3P9/3T3μ2	$6.6x10^7$	$1.0x10^7$	$2.2x10^7$
G2P9/3T3μ2	$6.4x10^7$	$2.0x10^7$	

In spite of this, plaques were picked from different passages in μ2 expressing cells and assayed on normal L929 and μ2 expressing cells for either infectivity in small volume culture or by plaque assay on 24 well dishes (Fig. 27). A total of 565 virus plaques from different passages and cell types were examined, some of them after repeated plaque purification (Table 4).

Table 4. Summary of plaques picked and assayed from different passages of T1, T3 and G2 virus. Numbers indicate number of plaques examined on 3 types of μ2 expressing cells.

Virus/Cell Type	L929/T1μ2	3T3/T3μ2	C127/T1μ2
T1P6	4	4	4
T3P6	8	4	40
G2P6	4	4	40
T1P7			
T3P7		3	
G2P7			4
T1P8	12	5	
T3P8		3	
G2P8	8	4	
T1P9		8	
T3P9		8	
G2P9		8	
T1P10			
T3P10	4*	100	
G2P10	30		
T1P11	4	4	
T3P11		54	
G2P11	4	4	
T1P12		4	
T3P12		92	
G2P12	51	40	

One virus plaque (* in table 4) #101-4 gave only one plaque on L929 but too numerous to count (TNTC) on L929 μ2 expressing cells (Table 5).

Table 5. Isolation of a putative M1 mutant. Comparison of the number of plaques produced by #101 before and after purification on L929 cells and μ2 expressing L929 cells.

Cell Type/ Plaques	L929	L929μ2
#101 (T3 P10/L929μ2)	9	40
#101-4	1	TNTC (too numerous to count)

However, in subsequent experiments no plaques could be recovered after infection with #101-4. It was concluded that the infectivity of the mutant was lost, possibly by storing the virus stock at +4°C (which is normally used for storage of reovirus) rather than at -20°C.

9.1.2 Generation of M1 mutants by UV irradiation and passage in μ2 expressing cells

Reovirus was treated with UV in order to generate mutants. Mutants would not be specific for the M1 gene but would be selected by passage and subsequent plaquing on μ2 expressing cells. The UV dose response curve of reoviral infectivity was determined by plaque assay following different doses of UV irradiation. One ml of T1P2 ($1.3x10^8$ pfu/ml) virus diluted 10 times with PBS was irradiated with UV from a distance of 15cm for 10, 20, 30 and 40 sec. At 40 sec. of UV treatment T1P2 had an infectivity of $2.5x10^6$ pfu/ml (2 logs reduction of titer).

UV treatment time was extended in order to obtain almost complete reduction in infectivity and maximize the likelihood of finding an M1 mutant. In the next experiment T1P2 and G2P2 were treated for 40, 80 and 120 sec. and passaged 2 times on μ2L929 cells. One passage was undiluted and the second was diluted 10x with PBS. Prior to each passage the harvested virus was treated with UV for the same length of time as P1 virus. T1P3 and G2P3 viruses were harvested and examined by plaque assay on L929 and μ2L929 cells. The plaque assay showed a trend towards lower titer with the length of UV treatment of the G2 virus on both types of cells. Type 1 reovirus titers showed some fluctuation following the treatment (Table 6).

Table 6. Analysis of the effect of UV irradiation on reovirus infectivity. T1 and G2 viruses irradiated with UV for 40s-2min. were passaged and assayed for plaque number on L929 cells and μ2 expressing L929 cells.

Virus/Time of UV irradiation (seconds)	Titer on L929 cells (pfu/ml)	Titer on L929μ2 cells (pfu/ml)
T1P3/40	$1.3x10^9$	$4.8x10^7$
G2P3/40	$1.2x10^9$	$6.2x10^7$
T1P3/80	$4.1x10^8$	$2.1x10^7$
G2P3/80	$5.4x10^8$	$1.5x10^7$
T1P3/120	$3.0x10^8$	$9.0x10^7$
G2P3/120	$2.7x10^8$	$1.2x10^7$

The titer was lower on μ2L929 than on L929 cells as observed before (Table 3). The difference in infectivity between virus plaqued on L929 cells and μ2L929 cells was greater in this experiment, 19-36 fold with the exception of T1P3/120, in which the difference was only 3 fold. Since viable virus could be recovered even after 2 min. of UV irradiation followed by passage in μ2L929, longer times (2-12 min) of exposure to UV light were used in the next experiment for T1P3/120sec. and G2P3/120sec. (viruses from the previous experiment irradiated for 2 min). The infectivity of T1P3/120 sec. and G2P3/120 sec. irradiated for 2-12 min. was assayed directly after UV treatment by plaque assay. G2P3/120 sec. reovirus retained its infectivity for up to 10 min. of UV treatment. The titer declined progressively on L929 and μ2L929 cells. T1P3/120 sec. virus was more susceptible to UV irradiation, losing the infectivity after 2 min. of treatment (Table 7). The difference in the plaquing ability of these two viruses on L929 and μ2L929 was within one log.

Table 7. Determination of UV dose sufficient to abolish T1 and G2 infectivity. G2 and T1 reovirus irradiation with UV from 2-12 min. directly assayed for infectivity on L929 cells and μ2L929. Note the higher susceptibility of T1 virus to UV compared with G2 reovirus.

Virus	Time of UV irradiation	Titer pfu/ml	
		L929	μ2L929
G2P3/120s	2	TNTC	TNTC
	3	TNTC	TNTC
	4	$2.2x10^3$	$3.8x10^2$
	5	$6.9x10^2$	$8.5x10^1$
	6	$9.0x10^1$	$2.0x10^1$
	7	$6.5x10^1$	$2.5x10^1$
	8	$1.3x10^2$	$2.0x10^1$
	9	$3.0x10^1$	-
	10	-	-
	11	-	-
	12	-	-
T1P3/120s	2	$1x10^3$	$2.0x10^1$
	3	-	-
	4	-	-
	5	-	-
	6	-	-

In spite of the fact that there were fewer plaques on μ2L929 cells than on L929 cells, viruses G2P3/120 sec. 4-12 min. and T1P3/120 sec. 2-5 min. were passaged once on μ2L929 cells followed by plaque assay. Three rounds of plaque purification were performed, however, none of these plaques had a growth advantage on μ2L929 cells.

The reason for why it was so difficult to select M1 mutant by passaging the virus on μ2 expressing cells remains obscure, especially because it was found in our lab that μ2L929 cells were able to complement the function of the mutated M1 gene in tsH11.2 (162). It can be assumed that it is more difficult to complement lethal mutants generated by serial undiluted passage than the ts mutant.