CHARACTERIZATION AND IDENTIFICATION OF THE REOVIRUS GUANYLYLTRANSFERASE

by

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A thesis submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

The reovirus guanylyltransferase has been studied in an effort to understand the transition from synthesis of capped mRNA by parental subviral particles (paSVP) to synthesis of uncapped mRNA by newly assembled progeny subviral particles (prSVP). The guanylyltransferase of paSVPs catalyzes the transfer of GMP from GTP to 5'-ppG-terminated oligonucleotides to form G(5')ppp(5')G cap structures. The reaction proceeds via a covalent, 5'-phosphoamide-linked enzyme-GMP intermediate. The [32P]GMP-labeled intermediate can be detected in virions, paSVP and cores (prepared by chymotryptic digestion of virions in vitro). Cores catalyze the transfer of GMP from GTP to purine nucleoside (5')-(p)pp-terminated acceptor molecules to yield cap structures with tri- and tetraphosphate bridges. The guanylyltransferase is also present in cores as a chymotryptic cleavage product of lambda 2, called lambda 2C, which is active in binding as well as transfer of GMP to form cap structures. Direct labeling of the guanylyltransferase with [32P]GMP has shown the activity to be associated with the lambda 2 protein of reovirus type 1 (RV1) and reovirus type 3 (RV3). Identification of the enzyme was facilitated by improving the separation of the reovirus lambda proteins on SDS-polyacrylamide gels. The order of migration of the three lambda proteins in the altered gel systems and in phosphate-urea polyacrylamide gels was determined by in vitro translation of the purified RV1 and RV3 dsRNA L gene segments in the presence of methyl mercury hydroxide. Identification of the guanylyltransferase as lambda 2 has provided an explanation of the structure and function of prSVPs.
Résumé

La guanylyltransférase de réovirus a été étudiée dans le but de comprendre la transition entre la synthèse d'ARNm avec structure chapeau (cap) par les particules-mères (paSVP) et la synthèse par les particules-filles (prSVP) d'ARNm sans chapeau. La guanylyltransférase des paSVPs catalyse le transfert de GMP à partir du GTP sur l'extrémité 5'-ppG des oligonucléotides pour former la structure chapeau G(5')ppp(5')G. La réaction passe par un intermédiaire enzyme-GMP comprenant un lien covalent 5'-phosphoamide. Cet intermédiaire [32P]GMP peut être détecté dans les virions, paSVP et structures cores (préparées in vitro par digestion à la chymotrypsine de virions). Les structures cores catalysent le transfert de GMP du GTP sur les nucléosides puriniques accepteurs se terminant par 5'-(p)pp pour produire des structures chapeau avec liens tri- ou tétraphosphate. La guanylyltransférase est également présente dans les cores sous forme d'un produit de clivage à la chymotrypsine de la protéine lambda 2, appelé lambda 2C, qui est actif à la fois pour lier et transférer le GMP pour former la structure chapeau. Le marquage de la guanylyltransférase à l'aide de [32P]GTP a démontré que l'activité est associée à la protéine lambda 2 des réovirus type 1 et type 3 (RV1 et RV3). L'identification de l'enzyme a été facilitée par l'amélioration de la séparation des protéines lambda sur gel de SDS-polyacrylamide. L'ordre de migration des trois protéines lambda dans ce système et dans les gels de phosphate-urée-polyacrylamide a été déterminée par traduction in vitro des segments génomiques L de RV1 et RV3 en présence de méthyl mercury hydroxide. L'identification de la guanylyltransférase comme étant lambda 2 procure une explication de la structure et fonction des prSVPs.
Dedication

To Mom and Dad

with love

Between two seas the sea-bird's wing makes halt,
Wind weary; while with lifting head he waits
For breath to reinspire him from the gates
That open still toward sunrise on the vault
High-domed of morning.

Swinburne.
Acknowledgements

I would like to thank Dr. Stewart Millward for giving me the opportunity to work with him and the freedom to learn from my own mistakes. The unstructured, congenial atmosphere he created encouraged independence and will be remembered with much fondness. His recent passing is felt with great sadness.

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Most of all, thanks to Ed, for his love and friendship, to my brother for keeping me on my toes and to my parents, for their continuous love and support since that fateful day 28 years ago.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Resume</td>
<td>11</td>
</tr>
<tr>
<td>Dedication</td>
<td>111</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>1v</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>5</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
<td>ix</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>Foreword</td>
<td>xiii</td>
</tr>
<tr>
<td>Chapter I INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Reovirus</td>
<td>4</td>
</tr>
<tr>
<td>Historical Perspective</td>
<td>4</td>
</tr>
<tr>
<td>Reovirus Particle Structure</td>
<td>6</td>
</tr>
<tr>
<td>Genome</td>
<td>7</td>
</tr>
<tr>
<td>Messenger RNA</td>
<td>9</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>10</td>
</tr>
<tr>
<td>Proteins</td>
<td>11</td>
</tr>
<tr>
<td>Outer Capsid Proteins</td>
<td>12</td>
</tr>
<tr>
<td>Core Proteins</td>
<td>14</td>
</tr>
<tr>
<td>Nonstructural Proteins</td>
<td>16</td>
</tr>
<tr>
<td>Reovirus Multiplication Cycle</td>
<td>17</td>
</tr>
<tr>
<td>Adsorption</td>
<td>17</td>
</tr>
<tr>
<td>Penetration and Uncoating</td>
<td>18</td>
</tr>
<tr>
<td>Transcription</td>
<td>19</td>
</tr>
<tr>
<td>Translation</td>
<td>20</td>
</tr>
<tr>
<td>Progeny Subviral Particles</td>
<td>23</td>
</tr>
</tbody>
</table>
Modification of Messenger RNA

Messenger RNA Cap Structures 25

Functions of the Cap 26

Stability 26

Processing of Pre-mRNA 27

Initiation of Translation 27

Formation of the Cap 29

Guanylyltransferases 30

The Reovirus Guanylyltransferase 31

Figures 1 to 3 35

Chapter II MATERIALS AND METHODS 38

Cells and Virus 39

Purification of Reovirus Oligonucleotides 41

Formation of an Enzyme-Guanylate Intermediate 42

Characterization of the Enzyme-Guanylate Bond 43

PEI Cellulose Thin Layer Chromatography 44

Polyacrylamide Gel Electrophoresis 45

Peptide Mapping 46

In vitro Transcription 46

Intermediate Formation with Reovirus-Infected Lysate Proteins 47

Effect of Nucleotides on the Formation of Intermediate 47

Transfer of GMP from Intermediate to Acceptor Molecules 48

Alkali Treatment of Cores 49

Purification of Reovirus dsRNA Genomic Segments 50

Methyl Mercury Agarose Gel Electrophoresis 51
Chapter III RESULTS

Characterization and Identification of the Reovirus Guanylyltransferase

Section 1 Intermediate Formation

Intermediate Formation
Nucleotide Specificity
Nature of the Enzyme-Guanylate Bond
SDS-PAGE Analysis of GMP-Labeled Cores
One-Dimensional Peptide Map Analysis
Parental Subviral Particle-Associated Enzyme
Solubilization of the Enzyme
Summary
Figures 4 to 13
Table I

Section 2 Transguanylylation

Nucleotide Release Experiments
Cap Formation by Core-Associated Enzyme
Cap Formation with Reovirus Oligonucleotides by Cores And Parental SVPs
Summary
Figures 14 to 18
List of Figures and Tables

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic Drawing of Reovirus Type 3</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>Composite Picture of Genomic dsRNA and Proteins of Reovirus type 3</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>5'-Terminal Structure of Reovirus Messenger RNA</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Optimization of Magnesium Concentration for the Guanylylation Reaction</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>Determination of the Optimal Temperature for the Guanylyltransferase and Effect of Pyrophosphatase on the Reaction</td>
<td>71</td>
</tr>
<tr>
<td>6</td>
<td>Kinetics of Incorporation of $[^{32}\text{P}]\text{NTP}$ into TCA Precipitable Material</td>
<td>72</td>
</tr>
<tr>
<td>7</td>
<td>CsCl Density Gradient Analysis of Labeled Cores</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>Analysis of the Guanylate Moiety of Labeled Cores</td>
<td>75</td>
</tr>
<tr>
<td>9</td>
<td>Polyacrylamide Gel Analysis of $[^{32}\text{P}]\text{GMP}$-Labeled Cores</td>
<td>76</td>
</tr>
<tr>
<td>10</td>
<td>One Dimensional Peptide Map Analysis of the $[^{32}\text{P}]\text{GMP}$-Labeled Core Proteins</td>
<td>77</td>
</tr>
<tr>
<td>11</td>
<td>Analysis of $[^{32}\text{P}]\text{GMP}$-Labeled Parental SVPs by Polyacrylamide Gel Electrophoresis</td>
<td>78</td>
</tr>
<tr>
<td>12</td>
<td>Transcriptional Activity of Parental SVPs Digested with Chymotrypsin</td>
<td>79</td>
</tr>
<tr>
<td>13</td>
<td>Intermediate Formation with Reovirus-Infected Lysate Proteins</td>
<td>80</td>
</tr>
<tr>
<td>14</td>
<td>Effect of Various Nucleotides on Enzyme-Guanylate Formation</td>
<td>87</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Analysis of Labeled Cores After Transguanylylation by Polyacrylamide Gel Electrophoresis</td>
<td>88</td>
</tr>
<tr>
<td>16</td>
<td>PEI Cellulose Thin Layer Chromatography of the Products Formed After Transguanylylation from Labeled Cores to Acceptor Nucleotides</td>
<td>89</td>
</tr>
<tr>
<td>17</td>
<td>PEI Cellulose Thin Layer Chromatography of the Products Formed After Transguanylylation from Labeled Cores to Reovirus Oligonucleotides</td>
<td>90</td>
</tr>
<tr>
<td>18</td>
<td>PEI Cellulose Thin Layer Chromatography of the Products Formed After Transguanylylation from Labeled Parental SVPs to Reovirus Oligonucleotides</td>
<td>91</td>
</tr>
<tr>
<td>19</td>
<td>Chymotrypsin Digestion of Labeled Top Component</td>
<td>105</td>
</tr>
<tr>
<td>20</td>
<td>Analysis of Alkali Treated Labeled Cores by Polyacrylamide Gel Electrophoresis</td>
<td>106</td>
</tr>
<tr>
<td>21</td>
<td>Analysis of dsRNA Genomic Segments of kV1 and RV3 by Polyacrylamide Gel Electrophoresis</td>
<td>107</td>
</tr>
<tr>
<td>22</td>
<td>Analysis of Purified dsRNA L Segments of RV3 by Polyacrylamide Gel Electrophoresis</td>
<td>108</td>
</tr>
<tr>
<td>23</td>
<td>Analysis of Purified dsRNA L Segments of RV1 by Polyacrylamide Gel Electrophoresis</td>
<td>109</td>
</tr>
<tr>
<td>24</td>
<td>Dot blot Hybridization Analysis of RV1 and RV3 dsRNA L Segments</td>
<td>110</td>
</tr>
<tr>
<td>25</td>
<td>Agarose Gel Analysis of RV3 dsRNA L Segments Under Denaturing Conditions</td>
<td>111</td>
</tr>
</tbody>
</table>
Figure

26 Analysis of In Vitro Translation Products of RV3 dsRNA L Segments by Polyacrylamide Gel Electrophoresis 112

27 Analysis of Lambda Proteins from RV1 and RV3 by Phosphate-urea Polyacrylamide Gel Electrophoresis 113

28 Analysis of Lambda Proteins from RV1 and RV3 on SDS-7.5%-Polyacrylamide Gels with Increased Crosslinking 114

29 Analysis of Lambda Proteins from RV1 and RV3 on SDS-6%-Polyacrylamide Gels with Reduced Crosslinking 115

Table

I Chemical Properties of the Enzyme-Guanylate Bond 74
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPI</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside 5'-triphosphate</td>
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<tr>
<td>NDP</td>
<td>nucleoside 5'-diphosphate</td>
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<tr>
<td>NMP</td>
<td>nucleoside 5'-monophosphate</td>
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<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>A</td>
<td>adenosine</td>
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<tr>
<td>C</td>
<td>cytidine</td>
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<td>U</td>
<td>uridine</td>
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<tr>
<td>I</td>
<td>inosine</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
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<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RV1</td>
<td>reovirus serotype 1</td>
</tr>
<tr>
<td>RV3</td>
<td>reovirus serotype 3</td>
</tr>
<tr>
<td>PaSVP</td>
<td>parental subviral particle</td>
</tr>
<tr>
<td>PrSVP</td>
<td>progeny subviral particle</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>kd</td>
<td>kilodaltons</td>
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<tr>
<td>bp</td>
<td>basepairs</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>S-10</td>
<td>supernate of a 10,000 x g centrifugation</td>
</tr>
</tbody>
</table>

Throughout this thesis, the letter \( u \) has been used to replace the symbol \( \mu \), representing \( 10^{-6} \) units, e.g. \( \mu l, \mu M, \mu g \).
Foreword

This project was initiated in collaboration with Dr. Helmut Zarbl. As such, some of the data contained in Chapter III, Sections 1 and 2 were obtained with his scientific and technical assistance. All experimental data presented in this thesis were obtained in whole or in part by myself. The findings are summarized in a manuscript entitled "The Reovirus Guanylyltransferase is L2 Gene Product, Lambda 2; Cleveland, Zarbl and Millward." This manuscript has been accepted for publication by the Journal of Virology.
CHAPTER I

INTRODUCTION
With the advent of antibiotics, mankind was finally able to prevent, control and cure bacterial infections. Unfortunately, we are still at the mercy of viruses. Although the actual pathology of a particular virus may only represent an inconvenience, as in the common cold, most viral infections haunt us for life. Shingles, polio and post-polio syndrome, hepatitis, herpes, retrovirus-induced cancer and now AIDS are only a few of the recurrent, crippling and deadly viral diseases that manifest themselves in man. Antiviral agents such as interferon have been useful in combatting some viruses but not all. Analogues of nucleotides can be used to mutate the quickly growing viral genome but their use is limited to controlling the infection once it has started. Antibodies have been raised to immunologically important surface antigens of viruses by inoculation with synthetic peptides and this approach to disease prevention seems promising.

However, alternative approaches are needed to fight viruses. The knowledge to develop these methods must come from an understanding of how the virus can infect and overcome a host cell. Knowledge of the virus’ life cycle will enable us to interrupt at a critical step.

My work focuses on reovirus, a relatively innocuous virus for humans. The life cycle of reovirus is an interesting one. It begins with production of capped mRNA by activated infecting particles. The capped mRNA is translated by the cell’s cap-dependent translational machinery. Later in infection, newly assembled progeny subviral particles synthesize uncapped mRNA which is translated in a cap-independent manner by an altered host translational machinery. Much work has been done to determine the nature of the alteration in the
host translational system. To complement these studies, I have focused on the switch from synthesis of capped mRNA to synthesis of uncapped mRNA.

Formation of the cap structure on the 5'-terminus of nascent mRNA is catalyzed by an enzyme called the guanylyltransferase. I have characterized this enzyme in reovirus and determined its genetic origin. Identification of the guanylyltransferase protein, in conjunction with previous data on the structure and transcriptional activity of progeny subviral particles, has enabled explanation of the lack of capping activity in these particles. To introduce this study, the structural and functional aspects of reovirus will be described and relevant aspects of mRNA modification and translation will be presented, especially with respect to the cap structure at the 5'-end of mRNA.
Reovirus

Research on reovirus has been extensive and a thorough review is contained in a book entitled The Reoviridae, edited by W. K. Joklik (1983), Plenum Press. This introduction is not meant to expound upon all previous work but is meant to bring out aspects of reovirus and mRNA capping pertinent to this study.

Historical Perspective

During the early 1950's, one of the priorities of research in health-related fields was the control and elimination of the crippling disease, poliomyelitis. During isolation of the causative agent for this disease, a group of other viruses were frequently found in the stool samples of patients. These viruses were called enteric cytopathogenic human orphan or ECHO viruses, for lack of a specific assignment to a disease in humans. ECHO virus group 10 was distinguished from the others primarily by its large particle size and pathogenicity. The ECHO 10 virus was renamed reovirus, an acronym for respiratory enteric orphan since these viruses were found in the respiratory tracts of patients and could not be associated with a disease (Sabin, 1957; 1959). Viruses found in Macaca monkeys, known as SV4, SV12, SV22, SV28, and SV59 and a reovirus-like agent in mouse hepatoencephalomyelitis could also be grouped with the reoviruses (Sabin, 1959; Rosen, 1962). The family Reoviridae has since grown to include, in addition to the genus orthoreovirus which includes the mammalian reoviruses described above, the orbi, rota, cypo, phytoreo and fiji viruses. The Reoviridae infect a wide range of hosts including vertebrates, insects and plants. Mammalian reovirus is not
generally associated with disease in humans although at least one report of a human death has been attributed to reovirus infection (Tillotson and Lerner, 1967). Mammalian reovirus is highly pathogenic for mice. Disease is also caused by other members of the Reoviridae, including rotavirus-associated diarrhea in most mammals and blue tongue virus-associated disease in sheep.

The Reoviridae are characterized by a genome of 10-12 segments of double-stranded RNA (dsRNA). Other viruses having genomes made of dsRNA are not included in the Reoviridae because of the presence of only 1 to 3 genome segments. These systems include the Birnaviridae, infectious pancreatic necrosis virus (IPNV), Tellina virus, oyster virus, Drosophila virus X, bacteriophage phi 6, mycoviruses and the virus-like yeast killer factor.

Mammalian reovirus is distinguished into three serotypes by properties of haemagglutination and antibody neutralization (Sabin, 1959; Rosen, 1960). The prototype for serotype 1 is the Lang strain. The prototype for serotype 2 is the Jones strain and there are two prototypes of serotype 3, the Abney and Dearing strains. The third serotype, reovirus type 3, bearing strain, has been used in most studies and was used for the majority of the studies presented in this thesis, with comparative work on serotype 1, Lang strain. Differences that can be noted between the serotypes include cell tropism, pathogenicity and specific nucleic acid and protein size differences (Ramig et al., 1977; Sharpe et al., 1978; Weiner et al., 1980).
Reovirus Particle Structure

The reovirus particle (Fig. 1) is a non-enveloped, double capsid icosahedron with a diameter of about 76 nm (Luftig et al., 1972). This value was obtained by electron microscopic methods and is smaller than the hydrated particle size which was estimated to be about 96 nm (Harvey et al., 1974). The particles have 12 projections known as spikes (Luftig et al., 1972), which correlates to having at least one spike for each of the 10 dsRNA genome segments. The existence of 12 genome segments in some members of the Reoviridae suggests that 2 genome segments may have been lost in mammalian reovirus during the course of evolution. The spikes are thought to be located at the 12 vertices of a particle exhibiting 5, 3 and 2-fold symmetry of rotation (Metcalf, 1982).

The outer capsid layer of protein is removed by uncoating of the particle in vivo within lysosomes or by digestion of virions in vitro with chymotrypsin. The particles that remain after digestion in vivo and in vitro are called parental subviral particles (pSVP) and cores, respectively. Cores have a diameter of about 52 nm as determined by electron microscopy (Luftig et al., 1972) and a hydrated diameter of 70 nm (Harvey et al., 1974). The proteins of the core are arranged in a symmetry similar to that of the virus particle. Removal of the outer capsid proteins also enhances visualization of the core-associated spikes by electron microscopy. The spikes have a 5 nm inner diameter, 10 nm outer diameter and are 5 nm in length. The core contains the 10 dsRNA genome segments which are always particle-associated within the infected cell (Silverstein and Dales,
1968; Chang and Zweerink, 1971). Virions also contain several thousand
oligonucleotides, of which the 5'-G-terminated molecules are released
from the viral particle when the outer capsid proteins are removed
(Carter and Lin, 1979). The core is extremely resistant to further
enzymatic digestion, and is stable for extended times at high
temperatures. Harsh conditions such as incubation in guanidine HCl,
high concentrations of urea or SDS are required to break up the core
proteins (Drayna and Fields, 1982b,c). Removal of the outer capsid
proteins of reovirus allows expression of a number of core-associated
enzyme activities which are required to synthesize capped mRNA from
the dsRNA genome (Faust et al., 1975; Furuichi et al., 1975a). These
enzymes have not been successfully removed from the viral particle in
an active form and may therefore be functionally dependent on the
structural integrity of the particle.

Genome

The first example of a dsRNA genome to be discovered was that of
reovirus. A number of properties showed that the genome of reovirus
was RNA in a double helical structure (Gomatos and Tamm, 1963). First,
staining of reovirus with acridine orange showed a pale green
fluorescence indicative of double-strandedness (Gomatos et al., 1962).
Under low salt conditions the genome was resistant to digestion by
DNases but not RNases. Subsequent purification of the genome allowed
further studies. The genomic RNA had a sharp melting point (Shatkin,
1965; Bellamy et al., 1967), was resistant to RNases under conditions
when the RNA might be duplex and was sensitive to digestion by RNase
III, a dsRNA specific nuclease. Formaldehyde, which binds to free
amino groups of nucleic acids, does not induce a shift in the absorbance maximum of reovirus genomic RNA (Gomatos and Tamm, 1963). The density of the genome was 1.61 g/cc in CsSO_4 and not 1.65 g/cc as is characteristic for ssRNA (Iglewski and Franklin, 1967). Determination of base ratios showed that U equaled A and G equaled C, and finally, X-ray diffraction analysis indicated a double-stranded structure (Arnott et al., 1967).

It was more difficult to demonstrate that the genome existed naturally as 10 discrete segments. It was argued that the procedures used to isolate reovirus particles and dsRNA could disrupt a single gene of the length predicted from measurement of total genomic RNA. Electron microscopic analysis of the genome showed three size groups of strands (Gomatos and Stoeckenius, 1964; Vasquez and Kleinschmidt, 1968). One of the strands of each gene segment had a 5'-diphosphate terminus, indicating that those termini did not arise by cleavage of a precursor (Banerjee and Shatkin, 1971). More importantly, it was shown that even before extraction of virus, twenty 3'-ends could be labeled (Millward and Graham, 1970). Another argument for a segmented genome is that transcripts are made in quantities inversely proportional to their length both in vivo and in vitro (Zweerink and Joklik, 1970). It has not been determined, however, whether the segments are linked by protein.

The 10 genome segments (see Fig. 2) can be separated by polyacrylamide gel electrophoresis into 3 size groups called large (approx. 3,800 bp), medium (approx. 2,200 bp) and small (approx. 1,200 bp), denoted by upper case letters L, M and S, with a collective
molecular weight of \(15 \times 10^6\) (Watanabe and Graham, 1967; Shatkin et al., 1968). The plus and minus strand of each genome segment are exactly complementary with the following general structure (Banerjee and Shatkin, 1971; Miura et al., 1974; Furuichi et al., 1975a,b; Chow and Shatkin, 1975; Muthukrishnan and Shatkin, 1975; Hastings and Millward, 1978);

\[
\begin{align*}
\text{mRNA} & : \quad \text{m}^7G(5')ppp(5')\text{GCUA----------UCAU(OH)}(3') \quad +\text{strand} \\
\text{OH}\text{CGAU----------AGUAGpp} & : \quad -\text{strand}
\end{align*}
\]

**Messenger RNA**

The dsRNA is transcribed conservatively into 10 mRNA species (Bellamy and Joklik, 1967; Skehel and Joklik, 1969). The mRNA species migrate on polyacrylamide gels in 3 size classes similar to those of the dsRNA segments and are called large, medium and small, denoted by the lower case letters l, m and s (Watanabe and Graham, 1967). The 5'-noncoding sequences of the mRNA are 15 to 33 nucleotides in length and do not show a tendency to form hairpin loops although the 3'-end can hybridize with the 5'-ends in most cases (Antczak et al., 1982). The 3'-ends of plus strand and messenger RNA are not polyadenylated (Stoltzfus et al., 1973). It has been shown that the plus strands of the genome are identical to the capped mRNA that is translated at early times during the infectious cycle (Hastings and Millward, 1981). Capped mRNA synthesized by paSVPs and cores can be translated into authentic proteins and the mRNA is packaged \textit{in vivo} and replicated to yield dsRNA-containing progeny SVPs (prSVP) (Schonberg et al., 1971). The dsRNA can be denatured and translated into authentic proteins (McCrae and Joklik, 1978). Each mRNA segment codes for at least one
primary protein (Both et al., 1975) and recent cloning (Cashdollar et al., 1984) and sequencing of the gene segments has made it possible to search the gene sequences for additional protein coding regions. So far, one such sequence has been found in the S1 gene, which codes for a 120 amino acid (14 kd) protein called sigma 1bNS or sigma s (Nagata et al., 1984; Cashdollar et al., 1985; Ernst and Shatkin, 1985; Jacobs et al., 1985; Sarkar et al., 1985). The AUG used to initiate this open reading frame is in a favorable consensus sequence whereas the 5'-proximal AUG used to initiate the larger 49 kd sigma 1 protein is not. It is therefore possible that a significant number of initiations occur at the second AUG, giving rise to the 14 kd protein (Kozak, 1982). In vitro translation of the S1 mRNA also demonstrates existence of two initiation sites and synthesis of a 14 kd protein in addition to the 49 kd protein (Cenatiempo et al., 1984; Jacobs and Samuel, 1985).

Oligonucleotides

Associated with reovirions are about 3,000 oligonucleotides which can be divided into two classes (Bellamy and Hole, 1970; Bellamy et al., 1972; Nichols et al., 1972; Stoltzfus and Banerjee, 1972). Two-thirds of the oligonucleotides have a 5'-G terminus and are 2 to 9 nucleotides in length. These oligonucleotides have the same 5'-terminal sequence as reovirus mRNA and are believed to be synthesized within the viral particle as a result of abortive transcription during the final phases of virus maturation (Bellamy et al., 1972; Yamakawa et al., 1981). The longer molecules of this class are terminated by a run of A residues and may represent
reiterative transcription or template-independent polymerization of ATP by a transcriptase which is undergoing conformational changes during particle maturation (Yamakawa et al., 1981). One third of the oligonucleotides in the viral particle consist of oligoadenylates of 2 to 20 nucleotides in length. Synthesis of oligo(A) by viral particles can be terminated by treatment of the particles with chymotrypsin and suggests that the outer capsid proteins may be regulating this altered activity of the core-associated transcriptase (Silverstein et al., 1974).

The reovirus oligonucleotides have mono, di and triphosphate 5'-termini. The 5'-G-terminated oligonucleotides are capped in vivo after uncoating of the virion and are released into the cytoplasm where they survive for at least 5 hours (Carter, 1977; Carter and Lin, 1979). No known function has been ascribed to the oligonucleotides and they are not required for viral infectivity (Carter et al., 1974). It can be speculated that release of thousands of capped oligonucleotides into the cytoplasm of the host cell may result in partial functional depletion of cap binding proteins and other initiation factors, thereby aiding the viral takeover of the cell.

Proteins

Each gene of reovirus codes for at least one protein. The coding assignments have been determined by in vitro translation of the purified dsRNA segments (McCrae and Joklik, 1978) as well as by use of intertypic recombinant virions. The latter method exploits the differences in mobility of each of the dsRNA gene segments and proteins of the 3 serotypes (Mustoe et al., 1978). Mustoe et al.,
(1978) did not determine the coding assignments for the L gene segments due to difficulties in distinguishing the three lambda proteins by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins can be separated by PAGE into 3 size groups called lambda, mu and sigma (see Fig. 2). Most of the proteins appear to have both structural (Loh and Shatkin, 1968; Smith et al., 1969) and enzymatic roles except for three nonstructural proteins mu NS, sigma NS and sigma 1bNS, which are found free in the cytoplasm of infected cells. One of the proteins present in virions is a cleavage product of mu 1 called mu LC (Zweerink and Joklik, 1970) and is the only reovirus protein known not to be blocked at the amino terminus (Pett et al., 1973). The nature of the blockage of the other proteins is not known.

The outer capsid layer of reovirus is made up of two abundant (mu LC and sigma 3) and one minor protein (sigma 1) and the core is made up of three abundant (lambda 1, lambda 2 and sigma 2) and three minor proteins (lambda 3, mu 1 and mu 2).

**Outer Capsid Proteins**

Sigma 1 is a minor, 42 kd protein coded by the S1 gene and is present in 24 copies per virion (two per spike). Antibody studies suggest that sigma 1 is located close to the spike protein, lambda 2 (Lee et al., 1961b). The functions of sigma 1 involve recognition and interaction of the virus with cell receptors and cell microtubules, haemagglutination (Weiner et al., 1978) and stimulation of antibody production (Weiner and Fields, 1977). The S1 gene product has also been implicated in the inhibition of host cell DNA replication (Sharpe and Fields, 1981). The role of sigma 1 in interaction of the virion
with the target cell suggests that it must be well exposed on the surface of the virion and it has been suggested to be located at the top of the spike with a coiled rod-like portion anchored inside the spike (Bassel-Duby et al., 1985). Sigma 1 is the reovirus protein that has diverged most between the three serotypes (Gaillard and Joklik, 1980; Lee et al., 1981a). A cDNA clone of the S1 gene has been expressed in E. coli under control of the lac promoter. The 47 kd fusion protein was immunoprecipitated with anti-sigma 1 antibodies, could bind to L-cell receptors and was a functional haemagglutinin (Masri et al., 1986).

The two abundant proteins of the outer capsid are mu 1C (550 copies per virion), coded by the M2 gene, and sigma 3 (900 copies per virion), coded by the S4 gene. These proteins appear to exist as a complex in a ratio of 1:2. Mu 1C is a 72 kd cleavage product of the carboxy terminus of mu 1, the primary translation product of the M2 gene (Zweerink and Joklik, 1970). Mu 1C may be polyadenylated, ADP-ribosylated, glycosylated and phosphorylated, although these modifications may actually be associated with mu 1 (Krystal et al., 1975, 1976; Carter et al., 1980). No function is known for an 8 kd fragment that is thought to be derived by cleavage of mu 1. Mu 1 exists free in the cytoplasm while most mu 1C is found in a complex with sigma 3 (Lee et al., 1981a). Sigma 3, 34 kd, has a high affinity for dsRNA and this property has been used to purify the protein by affinity chromatography (Huismans and Joklik, 1976). Sigma 3 also has the effect of reducing cellular RNA and protein synthesis (Sharpe and Fields, 1982). The sequence of the cloned cDNA of gene segment S4
suggests that sigma 3 is a hydrophilic protein (Giantini et al., 1984). Transcriptase activation by removal of the outer capsid proteins of virions in vivo results in cleavage of a 12 kd fragment from mu 1C, leaving the 65 kd fragment, delta. In vitro, mu 1C is completely removed by chymotryptic digestion of the outer capsid (Silverstein et al., 1972; Chang and Zweerink, 1971).

Core proteins

The proteins of the core include lambda 1, lambda 2, lambda 3, mu 1, mu 2 and sigma 2, which are arranged in capsomeres smaller than those of the outer capsid. The functional units of the core may be the spikes and their associated proteins since electron microscopy of transcriptionally active particles suggests that nascent mRNA is extruded through the spikes (Bartlett et al., 1974).

Lambda 1, encoded by genome segment L3, is 155 kd and present in 6 to 9 copies per spike unit. Iodination studies show that lambda 1 is labeled only after removal of the spike, suggesting that it is located beneath the spikes (White and Zweerink, 1976). There is no known function for lambda 1 although it is implicated in mRNA modification (Morgan and Kingsbury, 1981).

Lambda 2 is a 140 kd protein coded by genome segment L2. Lambda 2 also exists as a cleavage product, lambda 2C, in cores (this thesis, Shatkin et al., 1983) as well as in infected cell lysates (Lee et al., 1981a). Crosslinking studies show that pentamers of lambda 2 form the spike structure (Ralph et al., 1980). Lambda 2 is exposed on the surface of the virion as determined by interaction with monoclonal anti-lambda 2 antibodies (Hayes et al., 1981). The L2 gene of RV3 was
shown by intertypic recombinant analyses to dictate the capacity of RVJ to establish persistent infection and to generate defective interfering (DI) particles (Brown et al., 1983). The studies presented in this thesis demonstrate that lambda 2 is the reovirus guanylyltransferase. The relationship between the function of lambda 2 and its role in persistent infection and formation of DI particles will be discussed in Chapter IV.

Lambda 3, encoded by genome segment L1, is a 135 kd protein present in one copy per spike. Intertypic recombinant genome studies have shown that the pH optimum of the transcriptase segregates with the L1 gene segment (Drayna and Fields, 1982a). Although these results are highly suggestive, it remains to be unequivocally determined that the L1 gene product, lambda 3, is actually the transcriptase.

Mu 1, encoded by genome segment M2, is an 80 kd protein present in about 2 copies per spike. As discussed above, mu 1 also gives rise to the mu 1 cleavage product, mu 1C. The M2 gene segment products may be involved in regulation of reovirus transcription (Drayna and Fields, 1982a).

Mu 2, encoded by genome segment M1, is a 70 kd protein present in one copy per spike.

Sigma 2, encoded by genome segment S2, is 38 kd and is the most abundant protein of the core, present in 12 to 18 copies per spike. Sigma 2 may be on the inner surface of the core since iodination studies show that sigma 2 is iodinated to a lesser extent in intact cores than lambda 1 (White and Zweerink, 1976). The abundance of sigma 2 suggests that its role in the core may be mostly structural.
Nonstructural Proteins

Two of the nonstructural proteins are mu NS and sigma NS, 75 and 36 kd, coded by genome segments M3 and S3, respectively. A 70 kd cleavage product of mu NS, referred to as mu NSC, is present in amounts equal to mu NS (Lee et al., 1981a). These proteins are among the most abundant reovirus proteins produced in the cytoplasm.

Sigma NS has an affinity for ssRNA. This property has been used to purify the protein by affinity chromatography (Huismans and Joklik, 1976). It is speculated that sigma NS is a good candidate for an mRNA linking protein needed for assembly of 10 unique strands during particle formation (Stamatos and Gomatos, 1982). It was further shown that sigma NS binds to native as well as denatured UNA in vitro (Shelton et al., 1981) and is the sole protein component of particles with poly(C)-dependent RNA polymerase activity (Gomatos et al., 1980).

It was suggested that sigma NS in these particles could function as a replicase, to catalyze formation of dsRNA, since the particles can be used to synthesize poly(C):poly(C) using poly(C) templates. Expression of the cloned S3 gene segment in E. coli yields a protein which is identical to authentic sigma NS by peptide mapping. The protein can be immunoprecipitated and binds to ssRNA (Richardson and Furuichi, 1985).

A third nonstructural protein has been found recently which is coded by the smaller open reading frame of the S1 genome segment. This protein is about 14 kd and has been called sigma 1bNS, or sigma s (See description above).
Reovirus Multiplication cycle

The life cycle of reovirus has been studied largely by infection of mouse L-cells. L-cells are easily grown in large quantities in suspension cultures and reovirus can be obtained at high titers by infection of L-cells. At 37°C, infection of mouse L-cells with reovirus results in lysis of the cells at about 24 hours post-infection (Smith et al., 1969). At 31°C, lysis occurs after about 48 hours but with fewer cytopathic effects (cpe) than at 37°C.

Adsorption

L-cells in logarithmic growth phase are infected with 5 to 10 plaque forming units of virus per cell. Passage at higher multiplicities of infection promotes the assembly of viral particles called top component, which contain no RNA (Nonoyama and Graham, 1970; Nonoyama et al., 1970). The cell/virus mixture is incubated at 4°C for one hour, resulting in adsorption of 60 to 80% of the virus (Silverstein and Dales, 1968). Adsorption is performed at 4°C since it is possible to maintain the cells at high concentrations without loss of viability. Furthermore, while virions adsorb to cells at 4°C, no penetration occurs. Shifting the temperature up to 31°C or 37°C after adsorption therefore produces a more synchronous infection. The virus interacts with the cell via viral protein sigma 1 (Weiner et al., 1977, 1978; Lee et al., 1981b). The L-cell receptor for reovirus appears to be a 67 kd glycoprotein (Co et al., 1985) but does not seem to be like the glycoprotein erythrocyte receptors involved in reovirus haemagglutination (Armstrong et al., 1984).
Penetration and Uncoating

Virus particles enter the cell by a phagocytic process called viropexis (Dales, 1965; Dales et al., 1965). The particles within the phagocytic vesicles can be seen by electron microscopy to migrate toward the center of the cell and fuse with lysosomal vesicles by about one hour after penetration (Silverstein and Dales, 1968). Proteases inside these vesicles may be responsible for uncoating of virions.

An alternate mode of entry has been postulated (Borsa et al., 1979). Digestion of the virus particles by chymotrypsin in the presence of "non-facilitating ions" such as Na\(^+\) results in only partial uncoating of the outer capsid proteins (Borsa et al., 1973a,b). These particles, called intermediate SVPs, can infect cells directly, without apparent entry into vesicles. They also do not have an active transcriptase. Virus particles digested by chymotrypsin in the presence of "facilitating ions" such as K\(^+\) are fully uncoated and transcriptionally active. One can imagine a process whereby the Na\(^+\) rich environment outside the cell would be packaged into phagosomes and would promote partial uncoating by proteases to yield intermediate SVPs which could then pass through the vesicular membrane. After entering the K\(^+\) rich environment of the cytoplasm, the remainder of the outer capsid proteins would be removed to yield transcriptionally active paSVPs (Borsa et al, 1981). The ability of partially uncoated virions to penetrate membranes may explain how the particles could escape vesicles and enter the cytoplasm. It seems almost imperative that the particles do leave the vesicles since it is...
difficult to envisage mRNA synthesis in vesicles rich in phosphatases and nuclease. Uncoating of the virion by cellular enzymes demonstrates usage by the virus of cellular defence mechanisms. Uncoating of the virion in vivo (Chang and Zweerink, 1971; Silverstein et al., 1972) may occur sequentially with removal of sigma 3 first. The oligonucleotides are lost from the virion at this time (Carter and Lin, 1979). Mu 1C may be degraded in steps at which time the transcription-related enzymes are activated (Joklik, 1972; Carter, 1979; Ewing et al., 1985). The particle is still infectious until removal of sigma 1.

Transcription

Parental SVPs (paSVPs) and cores contain all the enzyme activities required to synthesize capped mRNA (Silverstein et al., 1972; Faust et al., 1975; Furuichi et al., 1975b). These include a dsRNA-depandant RNA polymerase or transcriptase which catalyzes end to end conservative transcription from the minus strand of the dsRNA genome (Borsa and Graham, 1968; Shatkin and Sipe, 1968; Watanabe et al., 1968a; Skehel and Joklik, 1969; Levin et al., 1970). The gamma phosphate at the 5'-terminus of the nascent message is removed by the nucleoside triphosphate phosphohydrolase (Borsa et al., 1970; Kapuler et al., 1970) to leave diphosphate-terminated mRNA which is a substrate for the guanylyltransferase. CMP is transferred from GTP to the nascent mRNA by the guanylyltransferase to form the cap structure G(5')ppp(5')GpC... (Furuichi et al., 1976; Furuichi and Shatkin, 1977b), which is subsequently methylated by viral and perhaps also host methyltransferases at two positions to give the final structure...
When cores are used in vitro to synthesize mRNA, transcripts of all ten gene segments are made simultaneously. In vivo, transcription of six of the ten gene segments is repressed. This stage of early transcription by paSVPs can be divided into pre-early and early events. Initially, transcripts are made only from gene segments L1, M3, S3 and S4 (Watanabe et al., 1968b; Millward and Nonoyama, 1970; Nonoyama et al., 1974). Two events may be involved in derepression of this state to allow transcription of the remaining genes. It was shown that the nonpermissive infection of mammalian L-cells with avian reovirus (Spandidos and Graham, 1976) or reovirus infection of L-cells in the presence of cycloheximide (Watanabe et al., 1968b; Lau et al., 1975) resulted in the same repressed state. Therefore, both translation of one or more of the pre-early messages and the presence of a factor from a compatible host cell may be required for derepression. A working hypothesis is that a host cell repressor interacts with the paSVP to restrict transcription. One of the early viral proteins, either lambda 3, mu NS, sigma NS or sigma 3, may be required to recognize and remove the host repressor. However, it is not known how these four specific genes escape repression.

Translation

The capped mRNA synthesized by paSVPs is translated by the host cap-dependent translational apparatus. There appears to be no specific enhancement of translation of viral versus cellular mRNA at this stage and translation probably occurs as a competition between the viral and cellular mRNA for ribosomes (Skup et al., 1981). Translation of reovirus mRNA in infected SC-1 cells was shown by Walden et al. (1981).
to involve competition when studied at a comparable stage post-infection. The only obvious difference between the viral and host mRNA is the lack of a 3'-poly(A) tail on the reovirus mRNA.

In addition to being involved in translation, capped reovirus mRNA also combines with newly synthesized viral proteins to form replicase particles. These particles are partially-formed, core-like structures which replicate the plus strand mRNA within the particle to yield dsRNA which serves as the genome (Watanabe et al., 1967; Schonberg et al., 1971; Zweerink et al., 1972; Sakuma and Watanabe, 1972). The particles continue to mature, by addition of proteins, into transcriptionally active progeny SVPs (prSVP). PrSVPs are morphologically different from paSVPs in that spikes are not detected by electron microscopy, and predictably, they contain reduced amounts of lambda 2 (Morgan and Zweerink, 1975). The mRNA capping enzymes of these particles are masked (Skup and Millward, 1980b) and as a result, prSVPs synthesize uncapped mRNA that is terminated by 5'-pGpC... (Zarbl et al., 1980b). As the infection proceeds, more and more prSVPs are assembled and more uncapped reovirus mRNA is synthesized. During the course of infection, there is a gradual decrease in translation of capped host and viral mRNA and a concomitant increase in the translation of uncapped viral mRNA (Skup and Millward, 1980a). Studies show that at late times post-infection, most polysomes contain uncapped reovirus mRNA (Skup et al., 1981).

Much work has been done to understand why cellular ribosomes would translate uncapped viral mRNA in preference to capped mRNA. This phenomenon has been thoroughly studied in other systems, the most notable being poliovirus-infected HeLa cells. The reduction of cap
binding proteins in these cells and resultant shutoff of translation of capped host mRNA allows translation of uncapped polio mRNA (Sonenberg et al., 1981b). Whereas uncapped polio mRNA can be translated in uninfected cell lysates, uncapped reovirus mRNA cannot (Skup and Millward, 1977), so there must exist an additional alteration in the host translational machinery that promotes translation of uncapped reovirus mRNA. Two alterations in reovirus-infected L-cells have been demonstrated. Cap binding proteins are functionally reduced in reovirus-infected L-cells at late times (Zarbl, Ph.D. Thesis, 1983), possibly decreasing translation of capped mRNAs. In addition, translation of uncapped reovirus mRNA is found to be stimulated at late times.

Work in the laboratory of Dr. S. Millward (Lemieux et al., 1984; Lemieux and Millward, submitted) has shown that the reovirus protein, sigma 3, may be responsible for the enhancement of translation of reovirus uncapped mRNA. Reovirus-infected L-cells were fractionated into S-200, ribosomal salt wash and ribosome fractions. The enhancing activity was greatest in the ribosomal salt wash, which was also enriched for sigma 3. Sigma 3 was purified by poly(I:C) affinity column chromatography according to the method of Huismans and Joklik (1976). Addition of purified sigma 3 to an uninfected L-cell translation lysate primed with uncapped reovirus mRNA stimulated translation of that mRNA. The cloned S4 gene obtained from Shatkin and coworkers has been expressed in L-cells under the control of the SV40 early promoter to yield a protein which comigrates with authentic sigma 3. The protein binds to ribosomes and translation of uncapped
reovirus mRNA is stimulated in lysates made from the transfected L-cells (Lemay and Millward, submitted).

Together, these mechanisms would not only decrease translation of host mRNA but would stimulate translation of the uncapped reovirus mRNA. Exclusion of capped reovirus mRNA from polysomes would also leave it free to be assembled into new prSVPs. Uncapped reovirus mRNA is not incorporated into particles (Furuichi et al., 1975b) suggesting that recognition of cap structures may be involved in packaging of capped mRNA into replicase particles.

**Progeny Subviral Particles**

PrSVPs are core-like particles that lack spike structures as seen by electron microscopy and have reduced amounts of lambda 2 as determined by phosphate-urea PAGE (Morgan and Zweerink, 1975). Mu NS and other non-core proteins are also transiently associated with prSVPs. Enzymes associated with prSVPs include the transcriptase and nucleotide phosphohydrolase activities already described for paSVPs. However, the capping activities are masked in prSVPs so that mRNA synthesized by these particles is uncapped and has a 5'-pG-terminus (Zarbl et al., 1980b). Absence of capping activity in these particles can be attributed to the absence of lambda 2, since it will be demonstrated in this thesis that lambda 2 is the reovirus guanylyltransferase. In addition, reovirus-infected L-cell lysates appear to contain a polynucleotide pyrophosphatase activity which may be responsible for removal of the 5'-terminal beta phosphate of nascent mRNA (Skup and Millward, 1980b; Zarbl et al., 1980b). The resulting 5'-pG-terminated molecules are not substrates for the
reovirus guanylyltransferase (Furuichi et al., 1976) or cellular guanylyltransferases (Venkatesan and Hoss, 1980).

Particles found in the infected cell at late times post-infection are diverse in composition, representing various stages of maturation (Zweerink, 1974; Morgan and Zweerink, 1975; Zweerink et al., 1976). During the final stages of infection, the prSVPs are transformed into virions by addition of the outer capsid proteins. Transcription is terminated by this maturation and nascent transcripts are aborted to yield the oligonucleotides found associated with virions (Bellamy and Hole, 1970; Nichols et al., 1972). Ultimately, reovirus multiplication results in lysis of the host cell and release of up to $3 \times 10^5$ newly synthesized viral particles as well as parental particles with reassembled outer capsid proteins (Silverstein et al., 1970; Chang and Zweerink, 1971; Astell et al., 1972).

The important steps in the takeover of host protein synthesis during the course of reovirus infection are the shift from production of capped to production of uncapped reovirus mRNA, and the concomitant alterations in the host translational system which allow translation of uncapped reovirus mRNA to the exclusion of both capped host mRNA and capped reovirus mRNA. This thesis focuses on the former aspect, the switch from synthesis of capped mRNA by paSVPs to synthesis of uncapped mRNA by prSVPs. In order to understand this switch, the capping enzyme or guanylyltransferase of paSVPs and cores was characterized and identified.
Modification of Messenger RNA

Eukaryotic mRNA synthesis begins in the nucleus by synthesis of a heterogeneous nuclear RNA (hnRNA) precursor which is subsequently modified and spliced to yield the functional mRNA that is transported into the cytoplasm. Modification consists of the addition of a 5'-terminal cap structure, internal methylation and 3'-terminal polyadenylation. Much work has been done on the mechanisms of modification and on their significance in the functioning of the mRNA in the cytoplasm. For the purposes of this thesis, I will focus on the modification at the 5'-terminus of mRNA, the cap structure (for a review see Banerjee, 1980).

Messenger RNA Cap Structures

The first demonstration of an unusual RNA 5'-terminus in small nuclear RNAs (snRNA) of Novikoff hepatoma cells (Reddy et al., 1972, 1974) was followed by further identification of RNA cap structures by other groups. These included the demonstration of caps on the RNAs of reovirus and cytoplasmic polyhedrosis virus (CPV) (Miura et al., 1974a,b). Definitive identification of the cap structure with its methylations came from work on reovirus, vaccinia virus and CPV (Faust et al., 1975; Furuichi and Miura, 1975; Furuichi et al., 1975a; Wei and Moss, 1975). Viruses have also played a fundamental role in the development of an understanding of the formation of cap structures and their function.

The presence of a cap structure at the 5'-end of mRNA is almost ubiquitous in eukaryotes except for the poly(A)+ mRNA of Hela cell mitochondria which has a 5'-ppA terminus (Grohman et al., 1878). There
are also several exceptions among the viruses. The RNAs of the picornaviruses, including EMC virus, poliovirus, and cowpea mosaic virus, are uncapped (5'-pU) but have a protein bound at the 5'-end (Hewlett et al., 1976; Lee et al., 1977; Daubert et al., 1978; Stanley et al., 1978; Golini et al., 1978). The protein is thought to be a primer for transcription (Nomoto et al., 1977) and is probably removed before translation (Ambrose et al., 1978; Dorner et al., 1981). Satellite tobacco necrosis virus mRNA is uncapped having a 5'-(-p)pApA terminus (Leung et al., 1979). In prokaryotes, mRNA is terminated with 5'-(-p)pGpG or 5'-(-p)pGpA. The 5'-penultimate nucleotide on capped eukaryotic mRNA can be any one of the four ribonucleotides (Cory and Adams, 1975) whereas in viruses, the penultimate nucleotide is usually a purine, either G or A, except for the existence of a GpppUp terminus in the early mRNA species of adenovirus (Hashimoto and Green, 1979). Although the penultimate base of eukaryotic mRNAs can be any base, the presence of a purine is believed to indicate capping of nascent mRNA whereas a pyrimidine indicates capping of cleaved mRNA (Schibler and Perry, 1976). Viral caps usually contain methylations at the N-7 and 2'-O positions.

Functions of the Cap

Three general functions have been associated with the presence of cap structures on mRNA. These deal with mRNA stability, processing of precursor mRNA and stimulation of initiation of translation.

Stability

Uncapped mRNA is degraded more quickly than capped mRNA in wheat germ extracts, L-cell lysates and Xenopus laevis oocytes but not in reticulocyte lysates (Furuichi et al., 1977). The presence or absence
of methyl groups in the cap structure had no effect on the stability of the mRNAs. Shimotohno et al. (1977) demonstrated that enzymatically decapped mRNAs, with 5'-pA termini, were degraded in a wheat germ extract but the capped mRNA homologues remained stable. Work of Skup and Millward (1980a) has shown that capped reovirus mRNA is stable in L-cell lysates but uncapped 5'-ppG-terminated reovirus mRNA is slowly degraded. The results were contrasted with those obtained in lysates from reovirus-infected cells, where both capped and uncapped mRNAs were stable although the 5'-terminal beta phosphate of the uncapped mRNA was hydrolyzed.

Processing of Pre-mRNA

Results of Konarska et al. (1984), Krainer et al. (1984) and Edery and Sonenberg (1985) suggest that the presence of cap structures on pre-mRNA promotes correct and efficient splicing of introns in an in vitro splicing system. The splicing of capped pre-mRNAs could be inhibited with cap analogues. Capped species of U1 snRNA have been shown to play a role in splicing by possible base pairing with the splice sites (Kraemer et al., 1984). Georgiev et al. (1984) have also reported that the presence of a cap at the 5'-end of histone precursor mRNA improves the efficiency of processing of the 3'-end.

Initiation of Translation

Much work has been done on the interaction of cap binding proteins (CBP) and other initiation factors with the cap structure and the way in which this interaction facilitates entry of capped mRNA into 40S initiation complexes (for review see: Shatkin, 1976; Filipowicz, 1978; Kozak, 1978; Banerjee, 1980; Shatkin, 1985).
Addition of cap structures to prokaryotic mRNA enables translation in eukaryotic cell-free translation systems (Paterson and Rosenberg, 1979). Enhancement of translation has been shown to be mediated by interaction of CBPs with the cap structure. A 24 kd CBP can be specifically crosslinked to oxidized cap structures and purified 24 kd CBP is found to stimulate initiation of translation of capped mRNA (Sonenberg et al., 1978, 1979). The 24 kd CBP interacts with 200 (220) kd and 46 kd proteins to form the CBP II complex (Grifo et al., 1983). In conjunction with eIF4A and eIF4B, the CBP II complex uses energy from ATP hydrolysis to interact with mRNA cap structures and 40S ribosomal subunits. The complex then travels in an ATP-dependent manner to the AUG initiation codon and combines with the 60S ribosomal subunit. Denaturation of secondary structure at the 5'-end of mRNA removes the need for ATP hydrolysis for ribosome binding but not for migration (Kozak, 1983). Continuing work by Sonenberg and coworkers has demonstrated that mRNA with reduced secondary structure appears to be less dependent on the presence of cap structures and on binding of some species of cap binding proteins for efficient translation initiation to occur (Sonenberg et al., 1981a; Sonenberg et al., 1982; Pelletier and Sonenberg, 1985).

Elucidation of the status of CBPs during translation was done largely by studying picornavirus-infected cells. Shutoff of host protein synthesis by poliovirus involves inactivation of host initiation factors which interact with the cap structure to mediate ribosome binding (Rose et al., 1978). Under these conditions, the uncapped polio mRNA can still be translated since it does not require
cap binding proteins for efficient initiation of translation. The 220 kd subunit of CBP II is cleaved in cells which have been infected with poliovirus and rhinovirus. Infection with encephalomyocarditis (EMC) virus does not result in cleavage of the 220 kd subunit although another type of inactivation could not be ruled out (Etchison and Fout, 1985; Hosenkis et al., 1985). Shutoff of host protein synthesis by poliovirus is much more rapid than by EMC virus infection (Jen et al., 1980).

In the case of reovirus infection of L-cells, mRNA synthesized late in infection is uncapped and the host translational system has undergone an alteration which allows translation of the uncapped reovirus mRNA. The alteration is currently being investigated in the laboratory of Dr. S. Millward. Results of Zarbl (Ph.D. Thesis, 1983) demonstrate a decrease in the level of all species of CBP. Lemieux et al. (1984; submitted) have demonstrated specific stimulation of translation of uncapped reovirus mRNA in L-cell lysates after addition of the reovirus protein sigma 3. Early in infection, translation of reovirus capped mRNA probably occurs in competition with the capped host mRNA (Walden et al., 1981). Thus, during reovirus infection of L-cells, three mechanisms may be involved in the takeover of the cell translational machinery; competition with cellular mRNA at early times, followed by virus-induced reduction of capped mRNA translation and concomitant stimulation of uncapped reovirus mRNA translation.

**Formacion of the Cap**

The mechanism of cap structure formation has been found to proceed by similar mechanisms in most eukaryotic systems studied. Synthesis of
cap structures on nascent mRNA molecules is catalyzed by the guanylyltransferase, which transfers GMP from GTP to 5'-'ppN termini of nascent mRNA. Capping occurs after removal of the gamma phosphate from the 5'-pppN terminus of nascent mRNA by a phosphohydrolase. The cap structure is then methylated at the N-7 position of the terminal guanine and the 2'-O position of the penultimate guanosine by methyltransferases to yield the cap structure shown in Figure 3. These enzymes are often found closely associated with each other structurally and functionally. There are two known exceptions to this scheme of mRNA capping. Influenza virus has developed a mechanism to scavenge the first 10 to 15 bases of capped cellular mRNA to use as a primer for viral transcription, thereby obviating the need for viral mRNA capping enzymes (Plotch et al., 1981). Vesicular stomatitis virus mRNA capping enzymes form cap structures by addition of GDP to 5'-monophosphate-terminated mRNA (Abraham et al., 1975). VSV is also unique in its methylation in that the 2'-O methylation of the penultimate base, adenosine, occurs before N-7 methylation of the capping guanine (Testa and Banerjee, 1977).

**Guanylyltransferases**

Guanylyltransferases have been studied in a number of organisms. Much work has been done on the vaccinia virus guanylyltransferase (Martin et al., 1975; Shuman et al., 1980; Venkatesan et al., 1980a; Shuman and Hurwitz, 1981; Roth and Hurwitz, 1984). These studies demonstrate that the capping reaction proceeds via a covalent 95 kd enzyme-GMP intermediate linked through a 5'-phosphoamide bond to lysine. The enzyme was found to be part of a 120 kd multifunctional
enzyme complex containing methyltransferase and triphosphatase activities. The guanylyltransferase of rat liver nuclei (Mizumoto and Lipman, 1979; Mizumoto et al., 1982; Yagi et al., 1983) is a 69 kd bifunctional enzyme associated with a 5'-triphosphatase activity. The guanylyltransferase was also studied as a covalent, phosphoamide enzyme-GMP intermediate. In HeLa cells (Venkatesan et al., 1980b; Venkatesan and Moss, 1980; Shuman, 1982; Venkatesan and Moss, 1982; Wang et al., 1982), the guanylyltransferase is 65 to 68 kd and was also studied as a covalent, phosphoamide enzyme-GMP intermediate. A similar enzyme was found in wheat germ (Keith et al., 1982) and calf thymus (Nishikawa and Chambon, 1982). The guanylyltransferases of Saccharomyces cerevisiae and Artemia salina were found to be about 44 kd in size and were associated with 5'-triphosphatase activities (Itoh et al., 1984; Wang and Shatkin, 1984; Yagi et al., 1984).

The Reovirus Guanylyltransferase

Study of the reovirus guanylyltransferase began indirectly in 1970 when a GTP:PPi exchange activity was found to be associated with reovirus cores (Wachsman et al., 1970). The 5'-terminal structure of reovirus mRNA (Fig. 3) was subsequently shown to be \( \text{m}^7 \text{G}(5')\text{ppp}(5')\text{GmpCpUp}... \) (Faust et al., 1975; Furuichi et al., 1975a). The mechanism of formation of the cap structure was then determined to be a multistep synthesis consisting of removal of the gamma phosphate from the 5'-end of nascent transcripts by the phosphohydrolase, transfer of GMP from GTP to form a cap structure by the guanylyltransferase and finally methylation at two sites by the methyltransferase activities (Furuichi et al., 1976). These steps were
shown to be separable (Furuichi and Shatkin, 1977b) and manipulation of the pyrophosphate concentration during in vitro transcription could be used to influence the yield of capped or uncapped mRNA (Furuichi and Shatkin, 1976). Whereas the capping and transcription events of mammalian reovirus are separable events, it was found that synthesis of cytoplasmic polyhedrosis virus (CPV) mRNA could not occur in the absence of cap formation (Furuichi, 1978) and that the reaction was stimulated by the presence of S-adenosyl methionine as well as S-adenosyl homocysteine. In reovirus, synthesis of mRNA is an activity associated with cores but it has been recently shown that the phosphohydrolase, guanylyltransferase and methyltransferase activities could be detected in intact virions (Yamakawa et al., 1982b). This again demonstrated the ability to dissociate transcription and capping events during reovirus mRNA synthesis. In vitro transcription by CPV was shown to result in formation of cap structures with both tetraphosphate and triphosphate bridges (Smith and Furuichi, 1982). Transcription in reovirus can be primed by addition of Gp$_4$G structures to the reaction in vitro (Yamakawa et al., 1982a). However, it was shown that capping does not occur when the gamma phosphate at the 5' terminus of nascent mRNA is not hydrolysable (Reeve et al., 1982). This result suggests that tetraphosphate cap structures are not formed by mammalian reovirus unless transcription and capping events are separated.

As a result of the difficulty in solubilizing enzyme activities associated with reovirus, none of the mRNA transcription-related activities have been purified. However, these activities were shown to
be associated with the reovirus lambda proteins when chemical
modification of lambda 1 and lambda 2/lambda 3 with pyridoxal
phosphate resulted in inhibition of transcription and capping events
(Morgan and Kingsbury, 1981). The initial demonstration of a
guanylyltransferase intermediate was made in vaccinia virus by Shuman
and Hurwitz (1981). They showed that incubation of the vaccinia virus
guanylyltransferase with [alpha-^32P]GTP in the absence of cap
acceptors resulted in formation of a covalent enzyme-GMP intermediate.
Their work has prompted further characterization of the reovirus
guanylyltransferase by allowing the enzyme to be studied without
purification of the protein from the core particle. Studies on the
identification and characterization of the reovirus
guanylyltransferase are presented in this thesis.

Some of the preliminary work contained in Chapter III, Sections 1
and 2 was presented as an abstract at the Canadian Federation of
Biological Societies and American Society for Virology meetings
(Cleveland, D. R., Zarbl, H. and S. Hillward. Identification of the
Abstract No. 244, Edmonton, Alberta, Canada, June 14 to 18, 1982, and
American Society for Virology, Ithaca, New York, USA, August 2 to 6,
1982). Results similar to those presented in Chapter III, Section 1
were also obtained independently by Shatkin and coworkers and were
presented at the First International Symposium on Double Stranded RNA
Viruses (Shatkin, A. J., Furuichi, Y., LaFiandra, A. J. and M.
Yamakawa. Initiation of mRNA Synthesis and 5'-terminal Modification of
Reovirus Transcripts. Frenchman's Reef, St. Thomas, U.S. Virgin
Islands, October 5 to 10, 1982) (the abstracts from this meeting were published in book form; See Shatkin et al., 1983). The data presented at these meetings were essentially similar but there were two important differences. Shatkin's group failed to demonstrate cap formation by the GMP binding protein and therefore could not conclude that it represented the reovirus guanylyltransferase. The \(^{32}\text{P}\)GMP-labeled protein was not rigorously identified. Nevertheless, on the basis of comigration of the labeled intermediate with stained proteins in a single gel system (SDS-polyacrylamide gel system of Laemmli (1970)), they tentatively identified the reovirus guanylyltransferase as lambda 2. At that time, using the same criterion, we tentatively identified the enzyme as lambda 1. Because of this discrepancy and because of the poor separation of the reovirus lambda proteins in the gels described above, extensive work was done in order to unequivocally identify the reovirus guanylyltransferase. This work is presented in Chapter III, Section 3 of this thesis.
Figure 1. Schematic Drawing of Reovirus Type 3.

The proteins of the outer capsid (O/C) and core are shown in their postulated locations in the double capsid structure of the virion. The core-associated spikes (λ2) are shown to extend through the outer capsid layer. Ten genomic segments of dsRNA are located within the core and the ssRNA oligonucleotides (about 3000 per virion) are represented by wavy lines.
REOVIRUS

76 nm

o/c

σ1

σ3

μ1c

λ1

λ2

λ3

μ1

μ2

σ2
Figure 2. Composite Picture of Genomic dsRNA and Proteins of Reovirus Type 3.

The ten dsRNA genome segments are shown as they migrate in a 5%-polyacrylamide-Tris Acetate-EDTA gel, stained with ethidium bromide and detected by fluorescence. The 3 size classes, large, medium and small, are labeled (L1-L3, M1-M3, S1-S4) and the approximate or exact (where known) lengths are given in base pairs (bp).

The proteins of reovirus (3 size classes called lambda, mu and sigma) are shown as they migrate in two commonly used gels, an SDS-7.5%-polyacrylamide gel (Tris-Glycine) and a phosphate-urea 7.5%-polyacrylamide gel. The inset is of an SDS-polyacrylamide gel made with twice the usual bisacrylamide crosslinking (see methods), electrophoresed to resolve the lambda proteins. Proteins are detected by Coomassie Brilliant Blue staining. The gene-protein coding correlations are indicated by lines (L1 codes for Lambda 3, L2 codes for lambda 2 etc.). Mu 1C is derived by post-translational cleavage of mu 1. Molecular weights (MW), quantity of each protein per virion and location in the virion (C=core, O/C=outer capsid) are given. The two non structural proteins mu NS and sigma NS are not associated with the virion and therefore are not detected in the gels. A third nonstructural protein, sigma 1bNS (not shown in diagram), is derived from a second open reading frame of gene segment S1.
REOVIRUS TYPE 3 - DEARING STRAIN

**dsRNA**
- ~3800 bp (L1, L2, L3)
- ~2200 bp (M1, M2, M3)
- 1416 bp (S1, S2)
- 1329 bp (S3, S4)
- 1190 bp (S4)
- 1196 bp

**Protein**

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**Gels**
- 7.5% SDS PAGE
- TRIS ACETATE EDTA
- TRIS GLYCINE UREA

**MW**
- 155 K
- 140 K
- 135 K
- 80 K
- 72 K
- 70 K
- 42 K
- 38 K
- 34 K

**Location**
- C
- O/C
Figure 3. 5'-Terminal Structure of Reovirus Messenger RNA.

The diagram shows the structure at the 5'-terminus of capped reovirus mRNA (above) in comparison to the 5'-terminal structure of uncapped pG-terminated reovirus mRNA (below). The cap structure contains an inverted 5' to 5' triphosphate bridge and methyl groups at the N-7 position of the capping guanine and the 2'-0 position of the penultimate guanosine.
capped mRNA

uncapped mRNA
CHAPTER II

MATERIALS AND METHODS
Cells and Virus

Mouse L-929 fibroblasts (L-cells) were obtained from Armand Frappier Institute, Laval, Quebec. Cells were maintained either in monolayer or suspension cultures in Minimum Essential Medium, Eagle modified (MEM) (Flow Laboratories Inc.) supplemented with 5% fetal bovine serum (FBS) (Flow Laboratories, Inc.), 292 mg/l L-glutamine and 25 µg/ml gentamicin (Schering Pharmaceuticals Ltd.).

Mammalian Reovirus Type 3 (RV3) (Dearing strain) was obtained from the American Type Culture Collection. Mammalian Reovirus Type 1 (RV1) (Lang strain) was obtained from the laboratory of Dr. Bernard Fields, Harvard Medical School, Boston, Mass. Infection of L-cells with RV3 was done according to Smith et al. (1969). Cells in suspension were maintained under conditions of logarithmic growth (between $0.2 \times 10^6$ cells/ml and $0.8 \times 10^6$ cells/ml) before being infected with reovirus. For a 2 liter infection, $2 \times 10^9$ cells were collected by centrifugation at 800 rpm for 8 min using a refrigerated TEC centrifuge. The cells were washed once with 1 liter of cold phosphate buffered saline, Dulbecco modified (PBS) (Flow Laboratories, Inc.) and resuspended in 200 ml of unsupplemented cold MEM. Ten plaque forming units (pfu) (approximately 50 virus particles per pfu) of RV3 or RV1 were added per cell and allowed to adsorb to the cells for 1 h at 4°C with slow stirring. The cells were then diluted to 2 liters with MEM supplemented with 2% FBS (heat-inactivated at 54°C for 30 min), L-glutamine and gentamicin and grown at 31°C for 42 h for RV3 or 34°C for 72 h for RV1 or until the cells showed 10%-30% cytopathic effects (cpe). Cpe were measured by non-exclusion of trypan blue.
stain. Cells were collected and frozen at -70°C until needed. Virus was purified according to Smith et al. (1969) by extraction with Freon and repeated isopycnic centrifugation on CsCl gradients (1.31 g/cc to 1.42 g/cc CsCl in 10TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)). The virus (1.36 g/cc) and top component (TC) (1.29 g/cc) were removed and dialysed against 2 changes of 100 mM Tris-HCl, pH 7.5, aliquoted and stored at -70°C. Calculations of virus concentration were based on the following values known for RV3; 1 A260 unit equals 2.1 x 10^{12} particles equals 185 ug RV3; Reovirus is 43% core (nucleic acid and protein) by weight (Smith et al., 1969).

For the production of RV3 labeled with \[^{35}S\]methionine, \(2 \times 10^9\) infected cells were collected at 20 h post-infection at 31°C, washed once with warm PBS and resuspended in: 550 ml MEM (minus methionine), 50 ml normal MEM, 2% heat-inactivated, dialyzed FBS, glutamine and gentamicin as above and 2 mCi \[^{35}S\]methionine. At 25 h post-infection, the cells were diluted to 2 liters with MEM supplemented with 2% heat-inactivated FBS, glutamine and gentamicin, and the infection harvested at 42 h post infection.

For the production of \[^{35}S\]methionine-labeled RV3 proteins in a cell lysate, cells from 10 ml of a 2 liter infection were collected at 20 h post-infection and resuspended in: 2.5 ml MEM (minus methionine), 2% heat-inactivated, dialyzed FBS, glutamine and gentamicin as above and 0.5 mCi \[^{35}S\]methionine, in a small glass scintillation vial. At 26 h post-infection, the cells were diluted in PBS and sedimented for 8 min at 800 rpm, washed and resealed for 5 min at 3000 rpm. The top of the pellet was rinsed gently with 50 ul of HKM buffer.
(10 mM Hepes, pH 7.5, 10 mM KOAc, 1.5 mM MgOAc). The pellet was resuspended in 150 ul of HKM buffer and adjusted to 1% Triton X-100 and vortexed for 10 min. The suspension was centrifuged for 15 min at 13,000 rpm, the clear supernatant fraction removed and aliquoted.

Reovirus cores were produced in vitro by digestion of virus with 50 μg of alpha-chymotrypsin/mg RV3/ml, 150 mM CaCl for 1 h at 45°C and purified by isopycnic centrifugation on CsCl gradients (density 1.31 g/cc to 1.48 g/cc) using a Beckman SW40 rotor at 34,000 rpm for 4 h at 4°C. The cores were dialysed against 2 changes of 100TE buffer (100 mM Tris-HCl, pH 7.5, 1 mM EDTA). Cores (2 A260/ml) were aliquoted and stored at -70°C.

Parental subviral particles (paSVP) (1.39 g/cc) were prepared by allowing a 2 liter RV3 infection of L-cells to proceed at 37°C for 7 h in the presence of 50 μg/ml cycloheximide. PaSVPs were purified as for virus, resuspended in 300 ul of 100TE buffer, aliquoted and stored at -70°C.

Purification of Reovirus Oligonucleotides

Purified reovirus (2 mg/ml) was digested with 0.2 mg/ml alpha-chymotrypsin for 1 h at 45°C. The cores were sedimented by centrifugation at 12,000 x g for 30 min using an Eppendorf centrifuge. The supernatant fraction was diluted to approximately 5 ml with 100TE buffer and centrifuged for 1 h in a Beckman SW50.1 rotor at 49,000 rpm, 4°C to remove residual cores. Proteins were separated from the oligonucleotides by 3 extractions with phenol followed by 2 ether extractions. The aqueous phase, containing the oligonucleotides, was divided into aliquots and dried by centrifugation under vacuum. Samples were stored at -20°C until needed.
Formation of an Enzyme-Guanylate Intermediate

Manipulations of radioactively labeled cores and paSVPs were performed with pipets and tubes coated with a solution of 5% dichloro-dimethylsilane in chloroform, dried, sterilized and further coated with bovine serum albumin. Renatured dialysis tubing was also precoated with bovine serum albumin.

Reaction mixtures contained paSVPs, RV3, RV1, purified viral cores (0.5-1.5 mg/ml) or top component (0.07 A_{260} units), 3 mM MgCl₂, 0.1 M Tris-HCl, pH 7.5, 20 units/ml of inorganic pyrophosphatase and approximately 1 uM [alpha-³²P]GTP (550-3000 Ci/mmol) or other radioactive nucleotides at similar specific activities. Reaction mixtures were incubated at 20°C for 2.5 h or for the times and at the temperatures indicated in the text. [Beta-³²P]GTP was made by Helmut Zarbl according to the method of Furuichi and Shatkin (1977a).

Transfer of [³²P] from [gamma-³²P]ATP to 5'-GMP was catalyzed by guanosine 5'-monophosphate kinase. The resulting [beta-³²P]GDP was further phosphorylated using pyruvate kinase to yield [beta-³²P]GTP.

For kinetic analyses of the guanylyltransferase reaction, aliquots of the reaction mixture were incubated in separate tubes and the reactions stopped at the appropriate times by the addition of 1 ml of cold 5% trichloroacetic acid (TCA) and 50 mM pyrophosphate. After 20 min on ice, samples were collected on Millipore filters (type HA, 45 μm) washed with 5% TCA, 50 mM pyrophosphate, dried and the radioactivity measured with an Intertechnique SL-36 liquid scintillation spectrometer using toluene-based scintillation cocktail.

Reaction mixtures were also analysed by isopycnic centrifugation on
CaCl gradients as described for core purification. Fractions were collected from the bottom of the gradients and Cerenkov radiation in each fraction was measured. The CsCl density of each fraction was measured using a Bausch and Lomb refractometer.

Characterization of the Enzyme-Guanylate Bond

Cores were incubated with [alpha-32P]GTP for 1 h at 20°C in 200 μl reaction volumes. The reaction was stopped by addition of 100 μl of 3X EMS buffer (1 mM EDTA, 2% beta-mercaptoethanol, 2% SDS). Solubilized proteins were separated from labeled GTP by gel filtration through 1.3 ml mini-columns of Sephadex G-25. Mini-columns were prepared in 1.5 ml Eppendorf tubes which were punctured at the bottom. The tube was filled with Sephadex G-25 over a glass wool plug, 1X EMS buffer containing 100 mM Tris-HCl, pH 7.5 added and the column pre-centrifuged at 2,000 rpm for 1 min in a clinical centrifuge. The sample was then applied to the column which was suspended in the top of a 13 x 100 mm glass culture tube and centrifuged at 2,000 rpm for 1 min. Aliquots of the eluate were then subjected to the following incubation conditions: 1) 0.1 N HCl, 100°C, 5 min; 2) 0.1 N NaOH, 100°C, 5 min; 3) 3.86 M hydroxylamine, pH 4.75, 37°C, 20 min; 4) 4 M sodium acetate, pH 4.75, 37°C, 20 min; 5) 0.2 M hydroxylamine, pH 7.5, 37°C, 20 min; 6) 0.5 mg/ml snake venom phosphodiesterase, 50 mM Tris-HCl, pH 8.0, 37°C, 1 h; 7) 0.5 mg/ml spleen phosphodiesterase, 50 mM Tris-HCl, pH 8.0, 37°C, 1 h; 8) 10 units/ml alkaline phosphatase, 50 mM Tris-HCl, pH 8.0, 37°C, 1 h. Appropriate controls were performed for each condition of incubation. Reactions were done in 1 ml final volumes. Reactions were
terminated by addition of 5% cold TCA and 50 mM pyrophosphate. Precipitates formed after 20 min on ice were collected on Millipore filters (type HA, 45 um) washed with 5% cold TCA, 50 mM pyrophosphate, dried and counted in toluene-based scintillation cocktail.

In order to analyze the label bound to core protein, [alpha-32P]GTP-labeled cores were incubated with 0.5 mg/ml of snake venom 5' phosphodiesterase, 50 mM Tris-HCl, pH 8.0 at 37°C for 1 h. The cores were removed by centrifugation as described. The supernatant fraction was prepared for analysis by PEI Cellulose Thin Layer Chromatography as described.

PEI Cellulose Thin Layer Chromatography

Supernatant fractions containing nucleotides to be analyzed were extracted three times with phenol with one back extraction, extracted twice with ether and applied to a 3 cm DEAE (diethylaminoethyl) column equilibrated with 5 mM triethyl ammonium bicarbonate (TEAB), pH 8.0. The columns were washed with 5 mM TEAB, pH 8.0 and eluted with 1.3 M TEAB, pH 8.0. Samples were evaporated to dryness three times by centrifugation under vacuum. The dry samples were finally resuspended in a small volume of distilled water and 5 ul aliquots spotted onto Machery-Nagel PEI Cel 300 thin layer chromatography plates (Randerath and Randerath, 1964). After spotting, plates were dried, washed with water and dried again. Chromatograms were developed in 1.6 M LiCl or in 1.0 M NaPhosphate, pH 3.5 (Cashel et al., 1969), the plates dried and autoradiography performed as described below. Non-radioactive markers were located using a long wavelength U.V. lamp.
Polyacrylamide Gel Electrophoresis

Proteins were analyzed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis as described by Laemmli (1970). Samples were solubilized in Laemmli sample buffer by boiling for 2 min. Gels were prepared using a stock solution of 29.2% acrylamide : 0.8% bis-acrylamide or as indicated in the text. Electrophoresis was for 3 h at 50 mA or overnight at 60 V with cooling or as indicated in the text. Stacking gels of 3.3% to 5% acrylamide were used and gels were electrophoresed in 4 litres of cooled running buffer, using a BioRad Protean gel electrophoresis apparatus.

SDS Phosphate-Urea gels (Zweerink and Joklik, 1970) were composed of 7.5% total acrylamide, 0.1 M NaPhosphate buffer, pH 7.2, 20 mM EDTA, pH 7.2, 6 M urea, 0.1% SDS, 0.06% ammonium persulphate (APS) and 0.1% TEMED. Solutions were dissolved at 20°C, filtered and degassed before addition of SDS, APS and TEMED. Upper gels were 5% total acrylamide in the same buffer as the resolving gels. The running buffer was composed of 0.1 M NaPhosphate buffer, pH 7.2, 20 mM EDTA, pH 7.2, and 0.1% SDS in 4 litres. Samples were solubilized in 1 volume of 2X sample buffer for a final concentration of 0.1 M NaPhosphate buffer, pH 7.2, 20 mM EDTA, 2% SDS, 20% glycerol and 5% beta-mercaptoethanol. Gels were electrophoresed at 50 mA for 24 h with recirculating buffer at room temperature.

After electrophoresis, gels were fixed and stained in 0.2% R-250 Coomassie Brilliant Blue stain, 50% methanol and 7% glacial acetic acid, for 45 min and destained overnight in 30% methanol. Gels were soaked for 1 h in 30% methanol, 20% glycerol and rinsed briefly before
being dried. Addition of glycerol was especially important to prevent cracking of the phosphate-urea gels during drying. Gels were dried on 3MM paper at 80°C under vacuum for 1.5 h and autoradiography was performed against Dupont Cronex X-ray film or Kodak XAR-5 X-ray film. Silver staining of gels was done using a BioRad Silver Stain Kit according to the directions in the kit.

**Peptide Mapping**

One dimensional peptide map analysis was done according to the method of Cleveland et al. (1977). The two major \(^{32}\text{P}\)-GMP labeled bands derived from labeled cores were excised from stained SDS-7.5%-polyacrylamide gels, equilibrated to Laemmli stacking-gel buffer and inserted into the wells of a SDS-15%-polyacrylamide gel. *Staphylococcus aureus* V.8 protease in quantities of 0.5, 1 or 5 µg in 20% glycerol was layered over the gel slices. Bromophenol Blue tracking dye in 10% glycerol was layered over the protease, and the proteins electrophoresed at 60 mA until the dye reached the interface between the stacking and resolving gels. The current was then turned off for 30 min to allow for partial digestion after which electrophoresis was completed at 40 mA. The gel was fixed, stained, dried and exposed to film.

**In Vitro Transcription**

RV3 (200 µg), paSVPs (30 µl of preparation described above) and chymotrypsin-treated particles were assayed for methylase activity and by inference, also for guanylyltransferase and transcriptase activities in reaction mixtures which contained; 50 mM Tris-HCl, pH 7.5, 10 mM MgCl\(_2\), 0.5 mg/ml bentonite, 2 mM each of ATP, UTP, CTP
and GTP, 20 mM phosphoenol pyruvate (freshly prepared), 25 units/ml pyruvate kinase (sedimented and resuspended in 50 mM Tris-HCl, pH 7.5) and 2.8 μM [3H-methyl]S-adenosyl methionine (82.6 Ci/m mole) (evaporated) in a final volume of 200 μl. The reactions were incubated at 45°C and aliquots of 40 μl were removed at various times and precipitated on ice in 5% TCA, 50 mM pyrophosphate for 20 min. Precipitates were collected on Millipore filters (type HA, 45 μm), washed with cold 5% TCA, 50 mM pyrophosphate, dried and counted in toluene-based scintillation cocktail. Messenger RNA synthesized during preparative transcription reactions was extracted with phenol and purified by Sephadex-G100 column chromatography in the presence of 0.5% SDS, 0.1 M NaCl, 1 mM EDTA and 5 mM Tris-HCl, pH 7.4. The void volume, containing mRNA, was precipitated in 0.15 M NaCl and 3 volumes of ethanol and stored at -20°C.

Intermediate Formation with Reovirus-Infected Lysate Proteins

S-10 lysates were prepared from uninfected L-cells and reovirus-infected L-cells as described (without label) and were then centrifuged at 200,000 x g for 30 min. The supernatant fractions (S-200) were dialysed against 100 mM KOAc, 10 mM Hepes, pH 7.4, 0.5 mM MgOAc and 1 mM DTT. 20 μl of S-200 was incubated with [alpha-32P]GTP in a standard guanylylation reaction and the proteins analyzed by SDS-polyacrylamide gel electrophoresis.

Effect of Nucleotides on the Formation of Intermediate

Purified cores were incubated with 3 mM MgCl₂, 0.4 μM [alpha-32P]GTP (550 Ci/m mole) and 15 units/ml pyrophosphatase in a final volume of 1 ml at 20°C. Aliquots of 20 μl were removed at 0,
5, 10 and 20 min and precipitated in cold 10% TCA. At 20 min, 100 ul of the reaction was added to each of 9 tubes containing 2 units pyrophosphatase and 0.25 umoles of either GMP, UDP, CDP, ADP, dGDP, GDP, GTP or ITP or 7 ul of water. Aliquots of 20 ul were removed at 25, 35, 45 and 60 min time points and precipitated in cold 10% TCA for 20 min. Precipitates were collected on Millipore filters, washed and counted.

Transfer of GMP from Intermediate to Acceptor Molecules

$^{32}$P-GMP-labeled cores were purified as described and incubated at 20°C in 100 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$, and 1 mM of either sodium pyrophosphate, GDP or GTP. Inorganic pyrophosphatase (10 units/ml) was added to the reactions containing GDP and GTP. After 15 min of incubation, cores were removed from the reaction mixtures by centrifugation and analyzed by PAGE. Supernatant fractions were extracted with phenol and with ether. Half of each sample was treated with alkaline phosphatase (40 units/ml) in the presence of 3 mM MgCl$_2$ at 45°C for 45 min. Samples treated with alkaline phosphatase were again extracted with phenol and prepared for thin layer chromatography and autoradiography.

Experiments using reovirus oligonucleotides as acceptors of GMP were performed with the quantity of oligonucleotides extracted from 0.75 mg of purified reovirus (see above). The aliquot of purified oligonucleotides was resuspended in 100 ul of distilled water and further purified by gel filtration through a Sephadex G-10 mini column previously equilibrated with water. The eluate was concentrated to 20 ul and added to a reaction mixture containing 150 ul of purified
[α-32P]GTP-labeled cores or parental SVPs, 3 mM MgCl₂, 100 mM Tris-HCl, pH 8.0 and 1.5 units inorganic pyrophosphatase in a final volume of 200 µl. The reaction mixture was incubated for 45 min at 20°C after which the cores or parental SVPs were removed by centrifugation and analyzed by PAGE. The supernatant fraction containing the oligonucleotides was brought to pH 8.0 with HCl and Penicillium nuclease Pl was added to a concentration of 0.4 mg/ml. The mixture was incubated for 4 h at 37°C and purified by extraction with phenol followed by extraction with ether. The aqueous phase was dried by centrifugation under vacuum and resuspended in 10 µl of water. Half of the sample (5 µl) was further digested by addition of 1 unit calf intestinal alkaline phosphatase (1 unit/µl), 0.5 µl of 100 mM Tris-HCl, pH 8.0 and incubated at 37°C for 1 h. Samples were then spotted onto PEI Cellulose thin layer chromatography plates and developed in 1.6 M LiCl.

Alkali Treatment of Cores

Alkali treatment of cores was carried out according to the method of White and Zweerink (1976). Cores were incubated with [α-32P]GTP in a standard guanylation reaction, sedimented by centrifugation and washed twice with 10TE buffer. The labeled cores were resuspended in 0.1 M NaPhosphate buffer, pH 11.8, incubated at 4°C for 15 min and then sedimented for 10 min using a Beckman Airfuge at 165,000 x g. The supernatant fraction was collected, neutralized with HCl and prepared for PAGE. The pellet was washed once with 10TE buffer and prepared for PAGE.
Purification of Repvirus dsRNA Genomic Segments

Purification of reovirus dsRNA was according to the method of McCrae and Joklik (1978). Reovirus (1 mg/ml) was adjusted to 1% SDS, extracted 3 times with 10TE-saturated phenol and then extracted with ether 4 times. The aqueous fraction was adjusted to 0.15 M NaCl and precipitated with 3 volumes of cold ethanol overnight at -20°C. The precipitate was collected by centrifugation for 30 min using an HB-4 rotor at 10,000 rpm. The pellet was rinsed with 70% ethanol, dried under vacuum and resuspended in 3X TAE sample buffer (final 10% glycerol, 0.17X TAE buffer, Bromophenol Blue and Xylene Cyanol). Ten times TAE stock buffer contained 0.4 M Tris, 0.2 M NaOAc and 10 mM EDTA, titrated to pH 7.8 with acetic acid. Polyacrylamide gels were composed of 5% total acrylamide, 0.1% TEMED and 0.03% APS in 1X TAE buffer. Gels were 3 mm thick and were polymerized with one long sample well. Pre-electrophoresis was carried out for 2 h at 120 V in 1X TAE running buffer. The running buffer was changed and the dsRNA sample was applied and electrophoresed for 48 h at 120 V with recirculation and cooling of the running buffer. Electrophoresis was monitored by viewing the dsRNA segments as light refracting bands in the gel. The running buffer was changed after the small and medium dsRNA segments had been electrophoresed off the bottom of the gel. After electrophoresis, the dsRNA segments were stained with 2.5 μg/ml ethidium bromide and visualized using a long wavelength U.V. lamp. Each L segment of dsRNA was excised and recovered by electroelution into dialysis tubing containing 2 ml of 0.5X TAE buffer. Electroelution was for 6 h at 150 V in 0.5X TAE running buffer with
cooling and recirculation of buffer. After electroleulont, the dsRNA in the dialysis bag buffer was collected and the bag washed twice with small volumes of 0.5X TAE buffer. The dsRNA solution was extracted 3 times with phenol saturated with 10TE buffer to remove acrylamide monomer, and 3 times with isoamyl alcohol to remove ethidium bromide. The aqueous phase was adjusted to 0.15 M NaCl and precipitated with 3 volumes of cold ethanol at -20°C overnight. The precipitate was collected by centrifugation using a Sorvall HB-4 rotor at 10,000 rpm for 30 min. The dsRNA was resuspended in water and quantified by absorption at 260 nm (50 ug dsRNA/A260). Aliquots were stored at -20°C. The quality of the purified dsRNA segments was verified by electrophoresis on TAE-5%-polyacrylamide gels and on methyl mercury agarose gels.

Methyl Mercury Agarose Gel electrophoresis

RNA denaturing gels were prepared according to the method of Bailey and Davidson (1976). E buffer was 50 mM boric acid, 5 mM sodium borate, 10 mM sodium sulfate, 1 mM EDTA titrated to pH 8.19 with NaOH. A 1.5% agarose solution in 30 ml of E buffer was prepared by boiling, cooled to 40°C and CH3HgOH was added to a final concentration of 5 mM. The gel was formed using the BioRad Mini-Sub Gel horizontal gel apparatus. The dsRNA samples were adjusted to 5 mM Vanadyl Ribonucleoside Complex (VRC), 1X E buffer and 0.0001% (w/v) bromophenol blue. Samples were heat shocked at 65°C for 2 min and adjusted to 5 mM CH3HgOH before electrophoresis at 70 V for 4 h with recirculating E buffer. Size markers consisted of Lambda phage DNA digested with the restriction endonuclease Hind III. Gels were stained
in running buffer containing 1 ug/ml ethidium bromide and 25 mM beta-mercaptoethanol for 0.5 h. RNA was visualized by trans-illumination with short wavelength U.V. light.

3'-Terminal Labeling of RV1 dsRNA

Labeling of the 3'-termini of dsRNA segments with [5'-32P]cytidine 3',5'-bis phosphate (pCp) using T4 RNA ligase was done according to the method of England et al. (1980). Reaction mixtures contained: 5 ug dsRNA segments L1, L2 or L3, 50 mM Hepes-KOH, pH 7.5, 20 mM MgCl2, 3.3 mM DTT, 10 ug/ml BSA, 10% (v/v) DMSO, 60 mM ATP, 170 uCi [5'-32P]pCp (3000 Ci/m mole) and 1.5 units T4 RNA ligase (24,000 units/mg) in a final volume of 15 ul. The reaction was incubated overnight at 4°C and the labeled dsRNA purified using Schleicher and Schuell Elutip-d columns. The columns were prewashed with 5 ml of 1 M NTE buffer (1 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 5 ml of 0.2 M NTE buffer (0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA). The ligase reaction mixture was diluted to 1 ml in 0.2 M NTE, applied to the column and washed first with 5 ml and then 10 ml of 0.2 M NTE. The dsRNA was eluted with 0.5 ml of 1 M NTE. A 5 ul aliquot of eluate was precipitated in 12% cold TCA with 200 ug yeast RNA carrier for 10 min on ice. The precipitate was collected on Millipore filters and radioactivity measured using toluene-based scintillation cocktail.

RNA Dot Blot Hybridization Analysis

Correlation of the dsRNA L segments of RV1 and RV3 was determined by hybridizing [32P]pCp-labeled denatured RV1 dsRNA L segments to cold denatured RV3 L segments which were blotted and baked onto
nitrocellulose filters (Millipore filters, type HA, 0.45 μm) (Thomas, 1980). Millipore filters were prewet in water and then in 20X SSC (1X SSC was 150 mM NaCl, 15 mM NaCl\(\text{O}^-\), pH 7.2) and laid on Whatman 3MM paper to soak up excess moisture. RV3 dsRNA segments L1, L2 or L3 (200 ng in 5 μl) were boiled for 3 min and adjusted to 14 mM CH\(_3\)HgOH, 10X SSC and spotted on the filters. The filters were baked for 2 h at 90°C and then soaked in 5 ml of prehybridization buffer (prepared by boiling 1 mg herring sperm DNA in 1 ml and diluting it with 6 ml of 20X SCP (2 M NaCl, 0.6 M Na\(_2\)HPO\(_4\), 10 mM EDTA, titrated to pH 6.2 with HCl), 5 ml of 10% Dextran Sulphate, 2 ml 10% Na N-lauroyl sarcosinate and 6 ml of water). After prehybridization at 65°C for at least 2 h, 3 ml of prehybridization buffer was removed and \([5'-\text{32P]}\text{PcP-labeled RV1 dsRNA probe was boiled and added to the remaining 2 ml of buffer. Hybridization was carried out at 65°C for 24 h. The filters were then removed and washed at 65°C with 4-5 changes of 50 ml wash buffer (0.1X SSC, 0.1% SDS, 0.1% pyrophosphate) for 2 h, dried and exposed to film.

Cell-Free Translation of Purified dsRNA

Highly efficient L-cell translation lysates were routinely prepared in our laboratory by Dr. Real Lemieux and Guy Lemay, according to the methods of Skup and Millward (1977) and Lemieux et al. (1984). L-cells were pelleted and allowed to swell in hypotonic buffer before being disrupted with a Dounce homogenizer. A 10,000 x g supernatant fraction was aliquoted, frozen and stored in liquid N\(_2\). These lysates were made from cells that had been starved for methionine in order to minimize the reduction in specific activity of
[35S]methionine added during translation. Lysates were treated with micrococcal nuclease prior to use to degrade the endogenous L-cell mRNA population.

In vitro translation mixtures in a final volume of 20 ul contained; 10 ul of nuclease-treated lysate, 4 ul of 5x master mix and 6 ul of RNA and/or water. The 5x master mix was made freshly each day from frozen stock solutions and contained: 0.6 M KOAc, 50 mM Hepes-KOH, pH 7.5, 2.5 mM MgOAc, 5 mM DTT, 0.25 mM spermidine, 50 mM creatine phosphate, 0.1 mM each of 19 amino acids (minus methionine), 2.5 mM GTP, pH 7.0, 2 mg/ml creatine phosphokinase (in 10 mM Hepes-KOH, pH 7.5, 50% glycerol) and 2.5 mCi/ml [35S]methionine (approx. 1000 Ci/m mole). Control incubations contained no RNA or 1 ug of heat-shocked total reovirus mRNA. The dsRNA segments were prepared for translation as follows: 1 ug dsRNA in 3 ul of water was combined with 1.2 ul of 0.1 mM EDTA, boiled for 4 min, and quickly frozen in liquid nitrogen. To the frozen RNA was added 1.8 ul of 5 mM CH3HgOH. Before addition of lysate and 5x master mix, the dsRNA/CH3HgOH was thawed and mixed carefully.

Translation mixtures were incubated at 30°C for 1 h. To monitor the reaction, aliquots of 2.0 ul were removed at 0 and 60 min, spotted onto 3MM filters and precipitated in cold 10% TCA for 10 min. The filters were boiled in 5% TCA for 10 min and washed with 5% TCA, ethanol, ethanol:ether (1:1) and finally with ether. Filters were dried and radioactivity counted in toluene-based scintillation cocktail. The remainders of the translation mixtures were prepared for analysis on polyacrylamide gels.
Materials

Radioactive nucleotides were obtained from New England Nuclear (Canada) Ltd. or ICN Biomedicals Canada Ltd. Inorganic pyrophosphatase and cycloheximide were from Sigma Chemical Company. Alpha-chymotrypsin and Staphylococcus aureus V,8 protease were from Miles Scientific. Snake venom phosphodiesterase and spleen phosphodiesterase were from Worthington Biochemicals. Calf intestinal alkaline phosphatase and creatine phosphokinase were from Boehringer Mannheim. All chemicals related to PAGE were from BioRad Laboratories. Penicillium nuclease P1, Vanadyl ribonucleoside complex and T4 RNA ligase were from Bethesda Research Laboratories. Methyl mercury hydroxide was from Alfa Ventron, Mass.
CHAPTER III

RESULTS

Characterization and Identification of the Reovirus Guanylyltransferase
It was shown by Shuman and Hurwitz in 1981 that the vaccinia virus guanylyltransferase could be studied as a labeled enzyme-GMP intermediate. The intermediate was formed by incubation of the viral enzyme with [alpha-$^{32}$P]GTP in the absence of cap-accepting substrates such as 5'-diphosphate-terminated mRNA. The method described by Shuman and Hurwitz was adapted and applied to reovirus in an effort to identify and characterize the reovirus guanylyltransferase.

The results obtained during this study will be presented in three sections. Section 1 contains data that demonstrate the GMP binding capacity of the guanylyltransferase of reovirus type 3 and characterization of the enzyme-guanylate intermediate of various reovirus particles. Section 2 describes transfer of GMP from the enzyme-guanylate intermediate to suitable substrates to form cap structures. Transfer of GMP from the intermediate to form cap structures verifies that the GMP binding protein described in Section 1 is indeed the reovirus guanylyltransferase. Section 3 deals more closely with identification of the reovirus protein associated with the guanylyltransferase activity.
Section 1
Intermediate Formation

Formation of a labeled guanylyltransferase-GMP intermediate in vaccinia virus was used to monitor purification of the vaccinia virus capping enzyme complex (Shuman and Hurwitz, 1981). Due to the apparent dependence of reovirus enzymes on particle structure, it was expected that the reovirus guanylyltransferase would have to be studied while associated with viral particles. Successful labeling of the particle-associated enzyme would obviate the necessity to purify it for further analysis, especially with respect to identification of the viral protein possessing the activity. Possible complications in labeling of the reovirion-associated enzyme involved steric hindrance due to the "closed" conformation of the particle and the presence in the particle of several thousand oligonucleotides. The oligonucleotides fall into two classes: the 5'-G-terminated oligomers and the oligoadenylates. The 5'-termini of both classes of oligonucleotides consist of variable quantities of mono-, di- and tri-phosphorylated structures (Nichols et al., 1972). Therefore, many of the oligonucleotides are potential substrates for the reovirus guanylyltransferase (Carter, 1977). To alleviate interference in studies of the kinetics of intermediate formation, virions were converted to transcriptionally active cores by removal of the outer capsid proteins with chymotrypsin. This process also releases the oligonucleotides from the particle. Cores derived by chymotryptic digestion of virions were purified by isopycnic centrifugation on CsCl
density gradients to separate the particles from the released oligonucleotides.

Intermediate Formation

Analysis of the first partial reaction of the guanylyltransferase,

\[ \text{GTP + ENZYME} \xrightarrow{\text{ENZYME-GMP + PPi}} \]

suggests that the presence of pyrophosphatase would enhance formation of labeled intermediate by removing PPi, thereby promoting the forward reaction. Thus, the standard guanylylation reaction mixture contained gradient-purified cores, \([\text{alpha}^{32}\text{P}]\text{GTP}, \text{Mg}^{++}\) and pyrophosphatase in Tris buffer. Formation of an enzyme-guanylate intermediate was monitored by the incorporation of \([^{32}\text{P}]\) into cold TCA-precipitable material. Incubation with various concentrations of MgCl₂ (Fig. 4) demonstrated that the guanylylation reaction had a broad magnesium ion optimum of about 2 to 4 mM. The effect of temperature on guanylylation is shown in Figure 5. Initially, reactions were carried out at 45°C, the optimum temperature for in vitro transcription by reovirus cores (Kapuler, 1970). Figure 5 shows that although there is a higher initial rate of incorporation of \([^{32}\text{P}]\) into TCA-precipitable material at 45°C, incorporation falls after 40 min. At lower temperatures, incorporation increases steadily for at least 3 hours. The apparent temperature dependence of the reaction might be explained by the instability of pyrophosphatase at higher temperatures. Inactivation of pyrophosphatase would result in accumulation of PPi and promotion of the reverse reaction. This appears to be the case since addition of pyrophosphatase (10 units/ml final concentration) to a reaction mixture that had reached a plateau at 45°C resulted in
further incorporation of label into TCA-precipitable material (Fig. 5, dotted line). Therefore, approximately 20 units/ml of pyrophosphatase was routinely added to the guanylylation reaction and reaction mixtures were incubated at 20°C for 2.5 hours.

Nucleotide Specificity

The specificity of the putative guanylyltransferase enzyme for GTP was tested by substituting various \[^{32}\text{P}]\text{NTPs}\) for \([\alpha^{32}\text{P}]\text{GTP}\) in the guanylylation reaction (Fig. 6). Incubation of cores with \([\alpha^{32}\text{P}]\text{GTP}\) resulted in almost linear incorporation of label into TCA-precipitable material for 25 min, whereas incubation of cores with \([\alpha^{32}\text{P}]\text{CTP}, \text{UTP, or ATP}\) or \([\beta^{32}\text{P}]\text{GTP}\) resulted in no incorporation. These results indicate that the reaction was specific for GTP and furthermore, that the alpha phosphate, and not the beta phosphate, of GTP remained associated with the TCA-precipitable material. As shown in Figure 6, addition of excess, unlabeled GTP after 2.5 min of incubation with \([\alpha^{32}\text{P}]\text{GTP}\) resulted in a rapid loss of label from TCA-precipitable material. The rapid decrease in detectable label is probably due to replacement of labeled GMP in the intermediate by excess unlabeled GMP. In this case, the loss of label is much more rapid than was the case in Figure 5 (45°C) and this will be discussed in Chapter IV.

In order to analyze the labeled, TCA-precipitable material, cores were collected from the above reaction mixtures by centrifugation, washed with TE buffer and subjected to isopycnic centrifugation on CsCl density gradients (Fig. 7). To avoid a smear of unincorporated \[^{32}\text{P}]\text{NTPs}\) across the gradient due to gradual loss of nonspecifically
associated NTP during banding, sedimented material was washed before application to the CsCl gradients. As a consequence of the washing, equal amounts of radioactivity were not applied to each gradient, although equal amounts of radioactivity and cores were used in each reaction mixture and all of the sedimented material from each reaction was analysed on the gradients. The gradients were fractionated and Cerenkov radiation in each fraction determined. The results show that radiolabel from [alpha-\(^{32}\)P]GTP, but not -ATP, -CTP or -UTP, was incorporated into material which banded at the characteristic density of cores (1.42 g/cc). Together, the data indicate that GMP was tightly associated with reovirus core particles. The nature of the labeled material at the bottom and top of the gradient containing [alpha-\(^{32}\)P]ATP was not determined, although it was assumed not to be associated with core proteins. This contention is supported by results of PAGE analysis of labeled cores presented in Section 2, Figure 15, demonstrating that none of the core proteins are labeled during incubation with [alpha-\(^{32}\)P]ATP.

**Nature of the Enzyme-Guanylate Bond**

The nature of the association between [\(^{32}\)P]GMP and reovirus cores was investigated by determining the chemical and enzymatic stability of the linkage. [\(^{32}\)P]GMP-labeled cores were purified by CsCl density centrifugation, solubilized with SDS, diluted to 1 ml to reduce the concentration of SDS and subjected to various enzymatic and chemical incubation conditions as described in Chapter II (Table I). After incubation, the amount of TCA-precipitable radioactivity was measured and compared to control values for each incubation condition.
A decrease in TCA-precipitable radioactivity relative to the control would indicate a susceptibility of the enzyme-GMP linkage to that incubation condition. Table I shows that the labeled GMP was not removed from the enzyme by alkaline phosphatase or by spleen 3'-phosphodiesterase but was removed by snake venom 5'-phosphodiesterase. The chemical tests show that the linkage was alkali stable, acid labile and was hydrolysed in neutral and acidic hydroxylamine. The nucleotide removed from labeled cores by snake venom 5'-phosphodiesterase was analyzed by PEI Cellulose thin layer chromatography. The sample was prepared for chromatography by phenol extraction and DEAE anion exchange chromatography. Figure 8 shows the autoradiogram of the PEI Cellulose thin layer chromatogram developed in two solvent systems, 1 M NaPhosphate, pH 3.5 and 1.6 M LiCl. The predominant $^{32}$P-labeled product comigrated with GMP markers in both cases, with low amounts of inorganic phosphate migrating with the solvent front. The inorganic phosphate may have arisen from breakdown of GMP during preparation of the sample. According to the chemical studies of Gumport and Lehman (1971) and Shabarova (1970), the data of Table I and Figure 8 suggest that GMP was covalently linked to the core protein through a 5'-phosphoamide bond. The same type of linkage has also been found in the guanylyltransferase intermediates from a variety of sources (Shuman and Hurwitz, 1981; Shuman, 1982; Venkatesan and Moss, 1982; Wang et al., 1982; Roth and Hurwitz, 1984).

**SDS-PAGE Analysis of GMP-Labeled Cores**

The protein component of cores to which GMP is bound was determined by separation of the labeled core proteins by
SDS-polyacrylamide gel electrophoresis (PAGE). Gradient purified, 
$^{32}$P]GMP-labeled cores were disrupted in SDS and analysed by 
electrophoresis on SDS-phosphate-urea 7.5%-polyacrylamide gels. As 
shown in Figure 9, the $^{32}$P label migrated as two major species in 
the region of the reovirus lambda proteins. Migration of the 
$^{32}$P]GMP-labeled bands was compared to the migration of reovirus 
proteins stained with Coomassie blue. In this gel system, lambda 1 
(155 kd) migrates as the slowest band while lambda 2 (140 kd) and 
lambda 3 (135 kd) comigrate as the next slowest band. The advantage of 
this gel system is that lambda 1 is well separated from lambda 2 and 
lambda 3 and the migration of the protein is not altered by addition 
or removal of GMP. This was shown by electrophoretic analysis of cores 
pre-incubated with unlabeled CTP or incubated with an excess of PPi to 
remove GMP that was already bound. Changes in protein concentration 
also did not detectably affect the migration of the viral proteins.
The disadvantage of phosphate-urea gels is the inability to separate 
lambda 2 and lambda 3. Comparison of the $^{32}$P]GMP-labeled proteins 
to stained markers in Figure 9 showed that the slowest labeled band 
(denoted P1) comigrated with lambda 2 and lambda 3 whereas the faster 
labeled band (denoted P2) (approximate apparent MW 125 k) did not 
comigrate with any of the lambda protein markers.

One-Dimensional Peptide Map Analysis

The slower and faster migrating $^{32}$P]GMP-labeled lambda 
polypeptides, P1 and P2, were analyzed to determine their 
relationship. P1 and P2 were cut out of a gel and the gel slices 
equilibrated with Laemmli stacking-gel buffer. One-dimensional peptide
mapping was performed according to the procedure of Cleveland et al. (1977) using three concentrations of *Staphylococcus aureus* V.8 protease (Fig. 10). It should be noted that only those peptides which have a $^{32}$P]GMP label will be detected by autoradiography. It would be impossible to analyse all of the peptides by protein staining because, at least for P1, the gel slice contained more than one protein species (lambda 2 and lambda 3) while it is expected that only one of those proteins was labeled with GMP. It would also be difficult to analyze the stained peptides of P2 since it was present in low amounts. The autoradiogram in Figure 10 shows that P1 and P2 had identical $^{32}$P]GMP-labeled peptides. Therefore, P1 and P2 are related, P2 probably being derived by cleavage of P1. Therefore, P2 was designated lambda C to signify "lambda cleavage product". Further identification of the P1 protein will be dealt with in Section 3 but for the moment it can be said that lambda 1 is not labeled, that P1 is either lambda 2 or lambda 3 and that lambda C (P2) is derived from either lambda 2 or lambda 3.

Although lambda C appears to be abundant because it is highly labeled, visual analysis of protein staining patterns on SDS-polyacrylamide gels indicates that lambda C is present in quantities similar to that of lambda 3 (12 copies per core). Quantification of the amount of $^{32}$P]GMP bound to cores was carried out in an effort to determine the number of GMP-binding sites associated with a core particle. Cores were incubated with $[^{32}$P]GTP at 20°C for 2.5 hours, sedimented and washed extensively. The amount of radioactivity per particle was measured and
gave a value of approximately 0.2 GMP molecules per core, or one out of five core particles labeled. Since some of the lambda proteins of reovirus are abundant, this value represents extremely low GMP binding. This is not entirely unusual in that enzyme intermediates are highly reactive and unstable forms of an enzyme. The structural constraints of the particle may also interfere with the labeling reaction. Alternatively, it is possible that most lambda molecules have only a structural role and are not enzymatically active. Nevertheless, even though the level of GMP-binding is low, the level of $[^{32}P]$ radioactivity associated with viral proteins is more than sufficient for further analyses.

**Parental Subviral Particle-Associated Enzyme**

Up to this point, all of the studies have been done using cores prepared by chymotrypsin digestion of virions in vitro. This raised the possibility that lambda C derives from chymotryptic cleavage of one of the lambda proteins. The significance of lambda C in the infected cell was investigated by determining whether the protein is present and functions as a GMP-binding protein in the in vivo equivalent of cores, namely parental subviral particles (paSVPs). PaSVPs can be isolated from L-cells infected with reovirus in the presence of cycloheximide, which inhibits protein synthesis and therefore the production of progeny subviral particles. PaSVPs result from in vivo uncoating of the infecting virions and are similar to cores in that they have all the enzyme activities necessary to synthesize capped mRNA (Silverstein et al., 1972; Galster and Lengyel, 1976). One apparent difference between cores and paSVPs is that the
latter retain a fragment of the outer capsid protein mu $\lambda C$, called delta. PaSVPs (1.39 g/cc) can be converted to cores (1.42 g/cc) by in vitro digestion with chymotrypsin. We therefore assayed for the presence of lambda $C$ in in vivo paSVPs and paSVPs treated in vitro with chymotrypsin. Particles were incubated in a standard guanylylation reaction with \([\alpha-^{32}P]GTP\), purified by CsCl density gradient centrifugation and the labeled polypeptides analysed by SDS-PAGE (Fig. 11). (Under commonly used conditions of SDS-PAGE described by Laemmli (1970), the 3 lambda proteins of reovirus are not well resolved from each other although P1 and P2 are well separated. This and other SDS-polyacrylamide gels shown in this thesis have been electrophoresed so that the reovirus sigma proteins have migrated off the end of the gel, allowing for greater resolution of the lambda proteins. Modifications to enhance resolving power of this gel system will be described in Section 3.) The labeling patterns were compared to the \([^{32}P]GMP\)-labeled proteins of cores. The results in Fig. 11 clearly demonstrate that label was incorporated only into the P1 protein of parental SVPs. When these particles were first digested with chymotrypsin in vitro, incubation with \([\alpha-^{32}P]GTP\) resulted in incorporation of label into P2 as well as P1. These results demonstrate that lambda $C$ was not detectable in paSVPs isolated from infected cells, but could be generated within these particles by in vitro digestion with chymotrypsin.

A few spurious \([^{32}P]GMP\)-labeled bands were seen to migrate faster than lambda $C$. These labeled bands did not comigrate with any reovirus marker proteins. Hence, their origin was surmised to be the
same as that of lambda C and they were not investigated. To ensure that chymotryptic digestion of the paSVPs did not cause general degradation of the particle to produce these faster-migrating labeled polypeptides, both chymotrypsin-digested and native paSVPs were examined for their activity in an in vitro transcription assay (Fig. 12). The activity of cores made by chymotryptic digestion of virions is also shown in comparison to the transcriptional inactivity of virions. Transcriptional activity was determined by measuring mRNA methylation as incorporation of [\(^{3}H\)]methyl groups from \([^{3}H\text{-methyl-}S\text{-adenosyl methionine into TCA-precipitable material. The amount of paSVPs used in the assay was lower than the amount of virions used, so that the two sets of data could only be compared among themselves and not between virus and paSVPs. The graph demonstrates activation of transcription after digestion of virions with chymotrypsin to form cores. PaSVPs, which are already transcriptionally active particles, did not undergo a significant alteration in activity after chymotrypsin digestion. Therefore, the production of cleavage products in chymotrypsin-digested parental SVPs was not a result of general paSVP breakdown.

**Solubilization of the Enzyme**

Efforts were made to identify a soluble form of the guanylyltransferase. Uninfected and reovirus-infected L-cell lysates were prepared at early times post infection. The lysates were sedimented at 200,000 \( \times \) g to remove virus and other particles. The supernatant fractions (S-200) were incubated with [\(\alpha\text{-}^{32}\text{P}\)]GTP and the labeled proteins analysed by SDS-PAGE (Fig. 13). Labeled proteins
of the uninfected S-200 and the infected S-200 were compared to the $[^{32}P]GMP$ labeled core proteins, P1 and P2. The autoradiogram shows that a number of cell-specific proteins were labeled in both lysates. The major band, migrating at an apparent MW of about 70 k, is in the same size range as the HeLa cell guanylyltransferase and may represent the L-cell guanylyltransferase. This was not further investigated. In the S-200 fraction from infected cells, there is, in addition to the cell-specific labeled bands, a labeled band migrating in the region of the lambda proteins which comigrated with P1 of cores. It is tempting to speculate that this band represents labeling of the soluble viral enzyme. However, the possibility remains that some residual viral particles were not completely cleared from the supernatant fraction even under these sedimentation conditions, making interpretation of the data in Figure 13 somewhat tenuous. Further attempts to label a soluble form of the guanylyltransferase were unsuccessful. These experiments are discussed in Chapter IV.

Summary

The data contained in this section have demonstrated the presence within reovirus particles of a nucleotide binding protein. The protein specifically binds GMP derived from GTP, through a covalent 5'-phosphoamide linkage. The $[^{32}P]GMP$-labeled reovirus proteins can be analysed by gel electrophoresis to show that label is associated with either lambda 2 or lambda 3 in paSVPs isolated from reovirus-infected L-cells. In cores, made in vitro by chymotrypsin digestion of virus, an additional polypeptide is labeled which is shown to be related to the labeled lambda 2 or lambda 3 of cores. The
additional labeled polypeptide is called lambda C for "lambda cleavage product". Tentative evidence is presented for an active, soluble GMP-binding protein.

The GMP-binding activity described in this section is thought to represent formation of an enzyme-GMP intermediate during the first partial reaction of the guanylyltransferase,

$$\text{pppG} + \text{ENZYME} \rightarrow \text{ENZYME-pG} + \text{PPi}.$$ 

To determine whether this is true, it is necessary to show that the purified enzyme-GMP intermediate can transfer GMP to suitable substrate molecules to form a cap structure via the second partial reaction of the guanylyltransferase,

$$\text{ENZYME-pG} + 5'\text{-ppGpC} \rightarrow G(5')\text{ppp}(5')\text{GpC} + \text{ENZYME}.$$ 

Experimental evidence for the above is presented in Section 2.
**Figure 4.** Optimization of Magnesium Concentration for the Guanylylation Reaction.

RV3 cores were incubated at 45°C with 1 uM \([\alpha-^{32}P]GTP\) (550-3000 Ci/mmol), 0.1 M Tris-HCl, pH 7.5 and varied concentrations of MgCl₂. Reactions were stopped after 20 min by precipitation on ice in 10% TCA. Samples were filtered, washed and the precipitated \([^{32}P]\) radioactivity measured.
Figure 5. Determination of the Optimal Temperature for the Guanylyltransferase and Effect of Pyrophosphatase on the Reaction.

RV3 cores were incubated with 1 μM [alpha-32P]GTP (550-3000 Ci/mmol), 0.1 M Tris-HCl, pH 7.5, 3 mM MgCl₂ and 20 units/ml pyrophosphatase at varied temperatures (20°C, 31°C, 45°C).

Aliquots of each reaction were removed at various times and precipitated on ice in 10% TCA. Samples were filtered, washed and the precipitated [32P] radioactivity measured. The dotted line indicates incorporation of [32P] into TCA-precipitable material after further addition of 15 units/ml pyrophosphatase to the 45°C reaction at 60 minutes.
Figure 6. Kinetics of Incorporation of $^{32}\text{P}]$NTP into TCA Precipitable Material.

RV3 cores were incubated at 45°C with 3 mM MgCl$_2$, 0.1 M Tris-HCl, pH 7.5, 20 units/ml pyrophosphatase and various $^{32}\text{P}$]-labeled NTPs (1 uM at 550–3000 Ci/mmol). Aliquots of each reaction were removed at various times and precipitated on ice in 10% TCA. Samples were filtered, washed and the precipitated $^{32}\text{P}$ radioactivity measured. Graphs show incubation with

[alpha-$^{32}\text{P}$]GTP (O),
[alpha-$^{32}\text{P}$]ATP (Δ),
[alpha-$^{32}\text{P}$]CTP (▲),
[alpha-$^{32}\text{P}$]UTP (■),
[beta-$^{32}\text{P}$]GTP (●),
[alpha-$^{32}\text{P}$]GTP with addition of cold, 1 mM GTP at 2.5 min (□).
Figure 7. CsCl Density Gradient Analysis of Labeled Cores.

RV3 cores were incubated at 45°C with 3 mM MgCl₂, 0.1 M Tris-HCl, pH 7.5, 20 units/μl pyrophosphatase and various [α-³²P]NTPs (1 μM at 550-3000 Ci/mmol) for 20 min. Reaction mixtures were sedimented, pellets washed and applied to preformed CsCl density gradients (1.31 g/cc to 1.48 g/cc). Gradients were centrifuged (34,000 rpm, 4 h, SW40 rotor) and fractionated dropwise from the bottom. Fractions were measured for CsCl density (●●●●) and Cerenkov radiation. Graphs show incubation with

[α-³²P]CTP (●●●●),
[α-³²P]ATP (■■■■),
[α-³²P]CTP (▲▲▲▲),
[α-³²P]UTP (□□□□).
Table I

Chemical Properties of the Enzyme-Guanylate Bond

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Total Acid Precipitable (k)cpm</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control (37°C, 60 min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/ml Spleen phosphodiesterase</td>
<td>128.0</td>
<td>100</td>
</tr>
<tr>
<td>0.5 mg/ml Venom phosphodiesterase</td>
<td>2.9</td>
<td>2</td>
</tr>
<tr>
<td>10 units/ml Alkaline phosphatase</td>
<td>128.0</td>
<td>100</td>
</tr>
<tr>
<td>B. Control (100°C, 5 min)</td>
<td></td>
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<tr>
<td>0.1 N NaOH</td>
<td>46.2</td>
<td>100</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>43.1</td>
<td>93</td>
</tr>
<tr>
<td>C. Control (37°C, 20 min)</td>
<td></td>
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<tr>
<td>3.86 M Hydroxylamine, pH 4.5</td>
<td>55.5</td>
<td>100</td>
</tr>
<tr>
<td>4.0 M Sodium acetate, pH 4.75</td>
<td>0.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>0.2 M Hydroxylamine, pH 7.5</td>
<td>42.4</td>
<td>76</td>
</tr>
<tr>
<td>0.8</td>
<td>2</td>
<td></td>
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</tbody>
</table>

* Part A was done with a different amount of starting material than Parts B and C.
Figure 8. Analysis of the Guanylate Moiety of Labeled Cores.

RV3 cores were incubated with \([\text{alpha-}^{32}\text{P}]\text{GTP}\), purified on CsCl density gradients, dialysed and incubated with 0.5 mg/ml snake venom 5'-phosphodiesterase, 50 mM Tris-HCl pH 8.0 at 37°C for 1 h. The cores were removed by centrifugation and the supernatant fraction prepared for thin layer chromatography. PEI Cellulose thin layer chromatograms were developed with A) 1.0 M NaPhosphate, pH 3.5 or B) 1.6 M LiCl. Chromatograms were dried and autoradiographed. Marker nucleotides were detected by fluorescence and are circled. Inorganic phosphate migrated with the solvent front.
A  
1M NaH$_2$PO$_4$  
pH 3.5

B  
1.6M LiCl

GMP  
GDP  
GTP  
ORIGINS
Figure 9. Polyacrylamide Gel Analysis of $^{32}\text{P}\text{GMP}$-Labeled Cores.

RV3 cores were incubated with [alpha-$^{32}\text{P}\text{GTP}$ in a standard guanylylation reaction, purified and the solubilized proteins analyzed by electrophoresis on a 7.5%-polyacrylamide phosphate-urea gel at 50 mA for 22 h. The largest labeled band (P1) comigrates with lambda 2/lambda 3. The smaller labeled band (P2) migrates ahead of the lambda proteins.

Lane 1: Coomassie blue-stained RV3 proteins as markers.
Lane 2: autoradiogram of $^{32}\text{P}\text{GMP}$-labeled core proteins.
Figure 10. One Dimensional Peptide Map Analysis of the 

\(^{32}\text{P}\)GMP-Labeled Core Proteins.

\(^{32}\text{P}\)GMP-labeled core proteins were separated by electrophoresis on an SDS-7.5% polyacrylamide gel. The labeled proteins P1 and P2 (see Figure 9) were excised and subjected to limited proteolysis by the method of Cleveland et al. (1977) using 0.5, 1 and 5 ug of Staphylococcus aureus V.8 protease in lanes a, b and c, respectively. \(^{32}\text{P}\)GMP-labeled peptides were detected by autoradiography as shown.
Figure 11. Analysis of $^{32}$P]GMP-Labeled Parental SVPs by Polyacrylamide Gel Electrophoresis.

RV3 parental SVPs (lane 2), RV3 parental SVPs digested with chymotrypsin (lane 3) or RV3 cores (lane 4) were incubated with [alpha-$^{32}$P]GTP in a standard guanylylation reaction and the solubilized labeled proteins analyzed by electrophoresis on an SDS-7.5% polyacrylamide gel for 3 h at 50 mA. Lane 1 shows Coomassie blue-stained RV3 proteins as markers. The two $^{32}$P]GMP-labeled bands are indicated as P1 and P2.
Figure 12. Methyltransferase Activity of Parental SVPs Digested with Chymotrypsin.

RV3 (VIRUS, open circles), cores (VIRUS + CH, closed circles), parental SVPs (paSVP, closed squares) or parental SVPs digested with chymotrypsin (paSVP + CH, open squares) were incubated with \[^{3}H\text{-methyl}\]-S-adenosyl methionine in standard transcription reaction mixtures at 45°C as described in Chapter II. Aliquots were removed at various times and precipitated on ice in 10% TCA. Samples were filtered, washed and the precipitated \[^{3}H\]-labeled material assayed. Incorporation of \[^{3}H\]methyl groups into TCA precipitable material was used as an indirect measure of transcriptional activity by assaying for formation of methylated cap structures.
Figure 13. Intermediate Formation with Reovirus-Infected Lysate Proteins.

Uninfected (U) and reovirus-infected (I) L-cell lysates were centrifuged at 200,000 x g and the dialyzed supernatant fractions incubated with [alpha-32P]GTP in a standard guanylylation reaction. The [32P]-labeled proteins were analyzed by electrophoresis on an SDS-7.5%-polyacrylamide gel at 50 mA for 3 h. Autoradiography shows the labeled cellular and viral proteins as compared to [32P]CMP-labeled core proteins (middle lane). Lambda and mu protein markers are included in the rightmost lane.
Section 2
Tranaguanylylation

Nucleotide Release Experiments

In order to demonstrate that the $[^{32}\text{P}]$GMP-labeled proteins represented a covalent enzyme-guanylate intermediate of the reovirus guanylyltransferase, formation of cap structures was examined by incubating the purified, labeled intermediate of cores and pSVPs with suitable acceptor molecules.

Various nucleotides were assayed for their ability to release the $[^{32}\text{P}]$GMP moiety from labeled cores (Fig. 14). This was done by incubating purified cores with [alpha-$^{32}$P]CTP in the presence of pyrophosphatase. At 20 minutes, the reaction mixture was divided into separate tubes and to each tube was added equal volumes and concentrations of either GMP, UDP, CDP, ADP, dGTP, GDP, GTP or ITP. An equal volume of water was added to a control tube. The effect of these additions on the further incorporation of labeled GMP into TCA-precipitable material was measured. The results shown in Figure 14 demonstrate an interesting pattern. Addition of GMP interfered with the labeling reaction compared to the control, but did not stop incorporation of $[^{32}\text{P}]$GMP altogether. In contrast, addition of UDP or CDP inhibited further incorporation of label as compared to the control, but did not appear to cause a release of label that had already bound to the cores. These in turn can be compared with the effects of adding ADP, dGTP, GDP, GTP or ITP. The latter group of purine nucleotides caused a definite release of label from cores.
The pattern of these results suggests specific interactions of the core-associated enzyme with these nucleotides in vitro.

Cap Formation by Core-Associated Enzyme

To take these tests a step further, the \(^{32}P\)GMP-labeled core intermediate was incubated in the presence of GDP, GTP and PPI, and the products of the reaction were analysed. The cores were sedimented for analysis by PAGE. The supernatant fractions were prepared for analysis by PEI Cellulose thin layer chromatography. Figure 15 is an autoradiogram of an SDS-polyacrylamide gel on which the cores sedimented from the reaction containing PPI were analysed. The two left-most lanes of Figure 15 show that label present in proteins P1 and P2 was completely removed after incubation with PPI. (Results of parallel analyses are also shown in this Figure. Incubation of cores with \([\alpha-^{32}P]\)CTP and -ATP did not result in incorporation of label into any reovirus core proteins as was determined in Section 1, Figures 6 and 7. Labeled material that did not enter the gel was not analyzed. This material seems to lose label in the presence of PPI and may represent nonspecifically labeled core aggregates).

The supernate containing the products formed during incubation of purified \(^{32}P\)GMP-labeled cores with pyrophosphate, GTP and GDP was analysed by PEI Cellulose thin layer chromatography (Fig. 16). Reaction supernates were purified by phenol extraction and DEAE Cellulose anion exchange chromatography. More than 95% of the radioactivity of the supernatant fraction was recovered after the purification steps. Half of each sample was digested with alkaline phosphatase (pme) to distinguish between free and blocked phosphate.
groups. For example, phosphates of GTP will be removed whereas the phosphates of GpppG cap structures will be protected. Figure 16 is an autoradiogram showing the labeled nucleotides purified from the reaction supernates before and after alkaline phosphatase digestion. Marker nucleotides were visualized with ultraviolet light and are circled. Small amounts of a pmn-sensitive spot co-chromatographing with GMP were found in each case, probably resulting from slow release of GMP from the labeled enzyme intermediate. Radioactive Pi produced by pmn-digestion migrated with the solvent front. Incubation of labeled cores with PPI yielded a pmn-sensitive spot comigrating with GTP. This reaction represents the GTP-PPI exchange activity first detected in reovirus cores by Wachsman et al., (1970). Incubation of labeled cores with GDP and GTP yielded pmn-resistant spots comigrating with GpppG and GppppG markers, respectively. These results demonstrate—formation of cap-like structures by purified labeled intermediate using nucleotides as analogues of the 5'-terminus of mRNA.

**Cap Formation with Reovirus Oligonucleotides by Cores and PaSVPs**

It still remained to be determined whether nascent mRNA molecules could also be capped by the enzyme-GMP intermediate. Uncapped 5'-ppG-terminated mRNA was synthesized in vitro using cores in the standard transcription reaction modified to synthesize uncapped molecules (Furuichi and Shatkin, 1976). The mRNA was purified by column chromatography and incubated with purified $^{32}P$GMP-labeled cores in an attempt to demonstrate cap formation on full-length, exogenous, uncapped mRNA. These experiments were unsuccessful. It may be that full-length mRNA cannot approach the active site of the
guanylyltransferase once it is dissociated from the core.

As an alternative, reovirus oligonucleotides were used as analogues of reovirus mRNA since it has been demonstrated that they are formed as the result of abortive transcription of the reovirus genome (Nichols et al., 1972; Zarbi et al., 1980a), and some have 5'-pppPurine termini which can be capped (Carter, 1977).

Oligonucleotides were isolated from chymotrypsin-digested virions and purified by column chromatography. Purified [32P]GMP-labeled cores were incubated with purified reovirus oligonucleotides. After reaction, the cores were removed from the mixture for further analysis. The supernates were collected and purified by phenol extraction and DEAE ion exchange chromatography. The oligonucleotides were prepared for thin layer chromatography by digestion with penicillium nuclease P1 (Shinshi et al., 1976). This treatment digests the oligonucleotide leaving intact cap structures with 3'-OH groups. Half of each sample was again treated with pme and the nucleotides analyzed by PLI cellulose thin layer chromatography using 1.6 M LiCl as solvent (Fig. 17). A control sample of labeled cores incubated in the absence of oligonucleotides showed some loss of pme-sensitive GMP into the supernatant fraction. The radioactive Pi produced by pme-digestion migrated with the solvent front. Incubation of labeled cores in the presence of oligonucleotides resulted in the formation of pme-resistant species comigrating with markers for GpppG and GpppA. More GpppG was formed than GpppA. This ratio may reflect the greater abundance of 5'-G-terminated oligonucleotides compared to oligoadenylates in the oligonucleotide preparation. An alternative
explanation is a greater affinity of the core enzyme for 5'-G-terminated oligonucleotides than for 5'-A termini. The cores that were removed from the above reaction were analyzed by SDS-PAGE (Fig. 15, right-most lanes). [32P]GMP label was lost from both the lambda and lambda C, demonstrating that lambda C is active in transfer of GMP to oligonucleotide acceptors as well as in GMP-binding.

The above experiment was repeated using in vivo derived paSVPs instead of cores. The paSVPs were labeled with [32P]GMP, purified and incubated with a sample of oligonucleotides identical to that used in the above experiment. The oligonucleotide products were prepared and analysed as above. The results are shown in Figure 18. Incubation of oligonucleotides with labeled paSVPs resulted in the formation of a pmr-resistant spot comigrating with the GpppG marker only. This result indicates that the guanylyltransferase of paSVPs specifically transferred GMP to 5'-G-terminated acceptor molecules and not to 5'-A termini. Since cores differ from paSVPs in that cores contain lambda C, and since [32P]GMP label is lost from both P1 and P2 after incubation of labeled cores with oligonucleotides (Fig. 15), one might surmise that the ability of cores to form cap structures with 5'-A-terminated oligonucleotides is a consequence of the presence of lambda C in the particle. Either lambda C is itself responsible for the activity or chymotryptic digestion alters the conformation of the particle so that the specificity of both labeled lambda proteins is reduced. The data of Figures 17 and 18 also do not show any evidence for tetraphosphate cap structures as were found in Figure 16 when labeled cores were incubated with GTP. Since the oligonucleotides of
reovirus include 5'-triphosphate termini, it appears that the guanylyltransferase of cores and paSVPs did not react with this subset of oligonucleotides.

Summary

It can be concluded from these transfer experiments that the GMP-binding activity described in Section 1 represents formation of an enzyme intermediate by the first partial reaction of the reovirus guanylyltransferase. The GMP moiety can be transferred from the intermediate to suitable acceptor molecules to form cap structures via the second partial reaction of the reovirus guanylyltransferase.

Partial identification of the $^{32}$P]GMP-labeled proteins was made in Section 1. Inadequate resolution of lambda 1, lambda 2 and lambda 3 in two commonly used gel electrophoresis systems prevented positive assignment of this activity to a specific reovirus polypeptide. In Section 3 of this thesis, the identity of the $^{32}$P]GMP-labeled reovirus protein, P1, will be conclusively determined.
Figure 14. Effect of Various Nucleotides on Enzyme-Guanylate Formation.

RV3 cores were incubated at 20°C with [alpha-^32P]GTP in a standard guanylylation reaction in a volume of 1 ml. After 20 min, 100 ul aliquots of the reaction were added to separate tubes containing pyrophosphatase and 0.25 umoles of either GMP (○), UDP (▲), CDP (□), ADP (●), dGDP (■), GDP (▼), GTP (❖) or ITP (●) or 7 ul of water (●). Aliquots were removed at various times and precipitated on ice in 10% TCA. Samples were filtered, washed and the precipitated [^32P] radioactivity measured.
Figure 15. Analysis of Labeled Cores After Transguanylylation by Polyacrylamide Gel Electrophoresis.

RV3 cores were incubated with [alpha-\(^{32}\)P]GTP, -CTP or -ATP, purified and half of the samples further incubated at 20°C with 1 mM pyrophosphate (PPi). The autoradiogram shows labeled core proteins before (-) and after (+PPi) addition of pyrophosphate. At the right are purified \([^{32}\)P]GMP-labeled core proteins before (-) and after (+oligos) incubation in the presence of reovirus oligonucleotides. Electrophoresis was in an SDS-7.5%-polyacrylamide gel at 60 V overnight.
Purified \([^{32}P]GMP\)-labeled cores were incubated with 1 mM pyrophosphate (PPi), GTP or GDP at 20°C for 15 min. The cores were removed by centrifugation and analyzed (see Fig. 15) and the supernatant fractions prepared for chromatography by phenol extraction. Half of each sample was digested with alkaline phosphatase (+pme) to distinguish between free and blocked phosphate groups. Samples were spotted and the chromatogram was developed with 1.0 M NaPhosphate, pH 3.5. Positions of marker nucleotides were detected by fluorescence and are circled. Inorganic phosphate (Pi) migrated with the solvent front. Chromatograms were dried and the spots detected by autoradiography.
Figure 17. PEI Cellulose Thin Layer Chromatography of the Products Formed After Transguanylation from Labeled Cores to Reovirus Oligonucleotides.

Purified [32P]GMP-labeled cores were incubated without (control) or with (+ oligos) reovirus oligonucleotides at 20°C for 45 min. The cores were removed by centrifugation and the supernatant fraction prepared for chromatography by digestion with Penicillium nuclease (0.4 mg/ml) at pH 6.0, 37°C for 4 h. The mixture was phenol extracted and concentrated by DEAE ion exchange chromatography. Half of each sample was digested with alkaline phosphatase (+pme) to distinguish between free and blocked phosphate groups. Samples were spotted and the chromatogram developed with 1.6 M LiCl. Positions of marker nucleotides were detected by fluorescence and are circled. Inorganic phosphate migrated with the solvent front. Chromatograms were dried and the spots detected by autoradiography.
Figure 18. PEI Cellulose Thin Layer Chromatography of the Products Formed After Transguanylylation from Labeled Parental SVPs to Reovirus Oligonucleotides.

Purified [32P]GMP-labeled parental SVPs were incubated without (control) or with (+oligos) reovirus oligonucleotides at 20°C for 45 min. The parental SVPs were removed by centrifugation and the supernatent fraction prepared for chromatography by digestion with Penicillium nuclease (0.4 mg/ml) at pH 6.0, 37°C for 4 h. The mixture was phenol extracted and concentrated by DEAE ion exchange chromatography. Half of each sample was digested with alkaline phosphatase (+pme). Samples were spotted and chromatograms developed with 1.6 M LiCl. Positions of marker nucleotides were detected by fluorescence and are circled. Inorganic phosphate migrated with the solvent front. Chromatograms were dried and the spots detected by autoradiography.
Section 3

Identification of the Reovirus Guanylyltransferase

Initial identification of the $^{32}\text{P}\text{GMP}$-labeled core protein was effected by electrophoresis on an SDS-7.5% polyacrylamide gel. Resolution of the lambda proteins was hampered when sufficient protein was applied to these gels for visualization by Coomassie blue staining (See also Mustoe et al., 1978). Therefore, reovirus-infected lysate proteins labeled in vivo with $^{35}\text{S}\text{methionine}$ were used as markers. Very low amounts of marker protein as well as $^{32}\text{P}\text{GMP}$-labeled core protein could then be applied to the gels in adjacent lanes and the labeled proteins could be detected by autoradiography. However, comparison of $^{32}\text{P}\text{GMP}$-labeled bands with $^{35}\text{S}\text{methionine}$-labeled lambda marker proteins gave confusing results. The GMP label comigrated with lambda 1 but it was found that the migration of the lambda marker proteins could be altered by changing the amount of lysate protein applied to the gel. The variable migration rates made confident identification of the guanylyltransferase difficult. This also demonstrated the importance of comparing radioactive profiles with the stained protein profile of that same lane.

Exclusion of Lambda 1 as the Guanylylated Protein

In phosphate-urea polyacrylamide gels, migration of the lambda proteins is not detectably altered by protein concentration. However, lambda 2 and lambda 3 comigrate as one band and cannot be separated. Lambda 1 migrates more slowly and is well separated from lambda 2/lambda 3. Analysis of the $^{32}\text{P}\text{GMP}$-labeled polypeptides on
phosphate-urea polyacrylamide gels (Fig. 9, Section 1) indicates that the label comigrated with lambda 2 or lambda 3. In conjunction with the good separation of lambda 1 from lambda 2/lambda 3, these results indicate that lambda 1 is not the guanylyltransferase. To further support this contention, additional evidence was obtained by using two previously reported procedures. The first involves chymotrypsin treatment of top component to cleave lambda 1 and the second involves alkali treatment of cores to solubilize lambda 2.

**Chymotrypsin Treatment of Top Component**

Smith et al. (1969) have shown that chymotrypsin digestion of top component (TC) results in removal of the outer capsid proteins. In addition, the core protein lambda 1 is cleaved into two smaller peptides of approximately 100 kd, called A and B, which migrate between the lambda and mu proteins on polyacrylamide gels. This cleavage does not occur when cores are generated from RNA-containing virions. The experiment of Smith et al. was repeated to determine whether cleavage of lambda 1 under these conditions would have an effect on the $^{32}\text{P}$-labeling pattern of the intermediate. TC was incubated with [alpha-$^{32}\text{P}$]GTP and then half the reaction mixture was digested with chymotrypsin. The proteins were analysed on a phosphate-urea polyacrylamide gel. Figure 19 is a composite of the radiolabeled and Coomassie blue-stained protein profile of $^{32}\text{P}$GMP-labeled TC before and after chymotrypsin digestion. Cleavage of lambda 1 to produce peptides A and B can be seen in the protein profile after chymotrypsin digestion. The radioactive profile of the same lanes shows no alteration in the labeling pattern. As
well, there was no radioactivity migrating at the bottom of the gel or in the region of peptides A and B. These data are in agreement with those of Figure 9 and demonstrate that $^{32}$PGMP is not associated with lambda 1. Therefore, lambda 1 is not the reovirus guanylyltransferase.

**Alkali Treatment of Cores**

White and Zweerink (1976) have reported that incubation of cores at pH 11.8, 4°C for 15 min results in the loss of spikes from the core surface, viral protein lambda 2 from the cores and a reduction in viral transcriptase activity. This study was done using phosphate-urea polyacrylamide gels at a time when the existence of lambda 3 was not known. Careful analysis of the data of White and Zweerink (1976) raised the possibility that lambda 3 may also have been lost after alkali treatment of cores. Therefore, this experiment cannot be used to distinguish between lambda 2 and lambda 3, but it can be used as additional evidence to support the exclusion of lambda 1 as the guanylyltransferase. As such, the experiment of White and Zweerink was repeated to determine the behaviour of the $^{32}$PGMP-labeled proteins under their conditions. Purified labeled cores were incubated at pH 11.8 as described and sedimented using a Beckman airfuge. The labeled proteins found in the pellet and supernatant fractions were analyzed on phosphate-urea 7.5%-polyacrylamide gels in comparison to labeled cores before incubation. Figure 20 is a composite of the stained protein pattern and the autoradiogram of labeled cores, the pH 11.8 pellet and the pH 11.8 supernate. The protein pattern shows a loss of lambda 2/lambda 3 from the pellet and the appearance of...
lambda 2/lambda 3 in the supernate. Analysis of the autoradiogram indicates that the \(^{32}P\)GMP label segregated with lambda 2/lambda 3 into the supernate and almost no label remained associated with the pellet after alkali treatment. These results indicate that the \(^{32}P\) label was associated with either lambda 2 or lambda 3, in agreement with the identification of the labeled band by migration in phosphate-urea polyacrylamide gels (Fig. 9, Section 1). In this particular experiment, lambda C was not very highly labeled with \(^{32}P\)GMP as seen in the autoradiogram of \(^{32}P\)cores, perhaps reflecting the spurious nature of the cleavage product. The labeled lambda C is even less apparent in the supernate although a labeled band migrating slower than sigma 1 may represent a digested form of labeled lambda C. This was not investigated further.

**Resolution of the Reovirus Lambda Proteins**

The remainder of this section will be devoted to describing attempts at improving the separation of lambda 2 and lambda 3 by altering gel electrophoresis conditions. In changing these conditions, the relative migration of the three lambda proteins needed to be re-established. This was done by translating the genetic information of the individual large reovirus genes *in vitro*. Since the mRNA strands which code for the three lambda proteins are difficult to separate, the corresponding double-stranded RNA genome segments were isolated instead. *In vitro* translation of dsRNA is difficult, especially for the L gene segments which are about 3,800 base-pairs in length (McCrae and Joklik, 1978), but was greatly improved by denaturing the strands with methyl mercury hydroxide. Methyl mercury
has been shown to improve translation of mRNAs (Payvar and Schimke, 1979).

The virus particles used in the following analyses included reovirus serotype 3 and reovirus serotype 1. The lambda proteins of RV3 and RV1 have been reported by Ramig et al. (1977) to have different relative mobilities in SDS-polyacrylamide gels. Lambda 1 and lambda 2 of RV1 migrate slower than those of RV3 in comparison to almost equal migration of lambda 3 in both serotypes. In the previous two sections, precautions were taken to study the guanylyltransferase as it exists in transcriptionally active particles. These particles included cores made in vitro and paSVPs formed in vivo. It was also important to study the GMP binding and transfer reactions in the absence of the reovirus oligonucleotides, which can act as cap acceptors. For most of the analyses that will be presented in this section, these precautions were unnecessary. The only requirement was that enough GMP-labeled protein be formed to allow for detection on gels. Although reovirions are not transcriptionally active, it was found that the guanylyltransferase could be labeled as an enzyme-GMP intermediate in these particles (see also Shatkin et al., 1983) as well as in top component particles (empty virions). No lambda C is detected in either of these particles. The advantage of using virions is that they are easily obtained and quantified.

The following will be presented in two parts. First, purification and in vitro translation of the dsRNA genome segments L1, L2 and L3 of RV1 and RV3 will be described. Then the gel systems used to distinguish between the three lambda proteins will be described. The
in vitro translation products of L1, L2 and L3 will be analysed on these gels and compared to the \(^{32}\text{P}\)GMP-labeled protein profiles of RV1 and RV3, cores and top component particles.

**Purification of RV1 and RV3 L gene segments**

Solubilized RV1 and RV3 virus particles were electrophoresed on an SDS-7.5% polyacrylamide gel. Figure 21 is a silver-stained profile of the dsRNA genome segments of RV1 and RV3. The fuzzy bands directly above the S3 gene segments represent lambda protein detected by the silver staining procedure. The gel was used to confirm that the genomes of the two serotypes used in this study correspond electrophoretically to serotypes 1 and 3 (Ramig et al., 1977). Total genomic dsRNA was purified from RV1 and RV3 by phenol extraction and the segments separated on a preparative 5%-polyacrylamide Tris-acetate-EDTA (TAE) gel. Electrophoresis conditions were used to maximize the separation of the L gene segments. The gel was stained with ethidium bromide and the segments visualized with long wavelength U.V. light. The L1, L2 and L3 gene segments were excised and eluted out of the gel slices electrophoretically. The segments were purified away from contaminating acrylamide monomer and ethidium bromide molecules by phenol extraction, isoamyl alcohol extraction and ethanol precipitation. The purity of the gene segments was confirmed by electrophoresis on TAE 5% polyacrylamide gels in comparison with the total genome segments. Figures 22 and 23 show the purified dsRNA segments of RV3 and RV1 respectively, as detected by ethidium bromide staining. Since the L2 and L3 gene segments of RV1 migrate very closely, some contamination of L3 with L2 can be seen in Figure 23.
This should not create a problem since the L2 gene preparation appeared to be pure. The L3 translation product could thus be identified by eliminating the L2 translation product which would be present in both reactions (see below).

Hybridization Analysis of dsRNA Segments

To ensure that the L1, L2 and L3 segments of RV3 corresponded genetically to the L1, L2 and L3 segments of RV1, nucleotide sequence homology was determined by dot blot hybridization analysis (Fig. 24). The three segments of RV1 were labeled at the 3'-terminus using [5'-32P]-cytidine 3',5'-bis phosphate and T4 RNA ligase. The segments were denatured and hybridized to the individual cold, denatured RV3 segments which had been baked onto nitrocellulose filter paper. The analysis was done in duplicate. The autoradiogram in Figure 24 shows that RV1 L1 hybridized to RV3 L1, L2 to L2 and L3 to L3 as expected. The data also indicate that the RV1 L3 segment was contaminated with L2 segment as was seen in Figure 23.

Agarose Gel Analysis of Denatured dsRNA Strands

The purified segments of RV3 were examined for the presence of single strand nicks and inter-strand crosslinking to ensure that the quality of the strands was adequate for in vitro translation. The L1, L2 and L3 segments of RV3 were denatured with methyl mercury hydroxide and electrophoresed in an agarose gel containing methyl mercury hydroxide (Fig. 25). Lambda phage DNA digested with Hind III restriction endonuclease was used for size markers. After electrophoresis, the gel was soaked in beta-mercaptoethanol to reverse methyl mercury hydroxide binding and allow for staining with ethidium
bromide. The stained bands were visualized using long wavelength U.V. light. Although there was a smear of RNA representing some degraded material, most of the RNA was found in a discreet band at approximately 3,800 bases for each segment. Double-stranded RNA of this quality translated well in vitro.

In Vitro Translation of dsRNA Genome Segments

Mouse L-cell translation lysates (Skup and Hillward, 1977) were used for in vitro translation of the purified L1, L2 and L3 genome segments. The lysates were prepared at various times in our laboratory by Dr. R. Lemieux and by G. Lemay. The purified dsRNA gene segments were prepared for translation by boiling for 4 minutes in the presence of EDTA, quick freezing in liquid nitrogen, followed by addition of methyl mercury hydroxide. The RNA was then allowed to thaw, mixed well and added to the translation mixtures containing $^{35}$S-methionine.

Typical results are shown in Figure 26, which is an autoradiogram of an SDS-7.5%-polyacrylamide gel showing the $^{35}$S-methionine-labeled translation products of RV3 dsRNA segments L1, L2 and L3, as well as total L-cell mRNA as compared to the in vivo $^{35}$S-methionine-labeled proteins of reovirus prepared from infected L-cells. Because the L gene segments are about 3,800 base pairs in length, reannealing during translation was a problem. Although a good yield of full-length translation product was obtained, many smaller polypeptides are seen on gels (see also McCrae and Joklik, 1978). These smaller products may derive from initiation at internal AUG codons or translation of degraded RNA strands or may represent aborted peptides resulting from reannealing of the plus and minus strands during translation.
Therefore, when analysing the results of in vitro translation of dsRNA, it is necessary to disregard the smaller bands and focus attention on the full-length products which comigrate with marker proteins. The translation products of segments L1, L2 and L3 are lambda 3, lambda 2 and lambda 1, respectively, as was determined for RV3 by McCrae and Joklik in 1978. In vitro translation of the dsRNA segments by McCrae and Joklik was done by denaturation of the dsRNA in 90% DMSO. Detection of the $^{35}$S-methionine-labeled lambda proteins by autoradiography required several months of exposure to film. In the experiments presented in this thesis, denaturation of the segments using methyl mercury hydroxide was very efficient and detection of the $^{35}$S-methionine-labeled lambda proteins required only overnight exposure to film. The gel shown in Figure 26 is an SDS-7.5%-polyacrylamide gel with regular cross-linking (29.2 g acrylamide : 0.8 g bis acrylamide). The close migration of the three lambda bands, which contain negligible amounts of protein in this case, demonstrated the need to develop PAGE conditions that would better resolve the lambda proteins.

**PAGE Analysis of Reovirus Lambda Proteins**

**SDS Phosphate-Urea PAGE.** The in vitro translation products described above and the $^{32}$P-GMP-labeled intermediate of the guanyltransferase of various particles were analysed by electrophoresis in a phosphate-urea 7.5%-polyacrylamide gel. The gel composition and electrophoresis conditions were essentially unaltered from the conditions used commonly in the reovirus literature (Zweerink and Joklik, 1970). Figure 27 is a composite of Coomassie blue-stained
protein profiles and autoradiograms of the labeled proteins. In part A of the figure, lanes 1, 2 and 3 show the $^{35}$S-methionine-labeled products of *in vitro* translation of RV3 dsRNA segments L3, L2 and L1 respectively. The arrowheads indicate the full-length products lambda 1, lambda 2 and lambda 3 respectively. These products comigrate with the lambda 1 and lambda 2/lambda 3 bands of RV3 protein markers (lane 6). $^{32}$P]GMP-labeled cores of RV3 were analyzed in lanes 4 and 5. Lane 4 shows the radioactive profile of the stained proteins in lane 5. The lambda C protein detected by staining and autoradiography did not comigrate with any peptides made by *in vitro* translation (lanes 1 to 3). The data in this figure confirmed the identification of the lambda bands in phosphate-urea gels, which was not done by McCrae and Joklik (1978). These same translation samples were further analysed in SDS-polyacrylamide gels in which the conditions of electrophoresis described by Laemmli (1970) were altered.

Part B of Figure 27 demonstrates that the guanylyltransferase of virions can be labeled with $^{32}$P]GMP. Lanes 1, 3 and 5 show the stained protein profiles of RV3, RV3 top component and RV1 respectively. Lanes 2, 4 and 6 are the corresponding autoradiograms of those lanes. The labeled band comigrated with lambda 2/lambda 3 in each case. No labeled lambda C is detected in these particles.

**SDS-PAGE in Gels with Increased Crosslinking.** The conditions of Laemmli (1970) were used with the following changes. Resolving gels of 7.5% acrylamide and stacking gels of 3.3% acrylamide in a ratio of 28.4 g acrylamide : 1.6 g bis acrylamide (2 times usual crosslinking) were used. Electrophoresis was for 6 hours at 50 mA (Figure 28).
Part A of Figure 28 is an autoradiogram of a polyacrylamide gel with increased crosslinking showing the \(^{35}\text{S}\)methionine-labeled products of in vitro translation of dsRNA segments L1, L2 and L3. Lanes 1, 2 and 3 show RV1 gene products lambda 3, lambda 2 and lambda 1, respectively. Lanes 4, 5 and 6 show RV3 gene products lambda 3, lambda 2 and lambda 1, respectively. In both cases, the proteins migrated in the order determined by McCrae and Joklik, 1978, namely lambda 1, lambda 2 and lambda 3 (largest to smallest). In lane 3, two lambda proteins are detected, the faster one comigrating with lambda 2 in lane 2. This protein is detected due to the contamination of RV1 L3 gene segment with L2 gene segment as determined in fig. 23. In lane 6, two bands are also seen, although the faster migrating band does not quite comigrate with lambda 2 in lane 5. Since the RV3 L3 gene segment was not determined to be contaminated with L2 gene segment, the faster migrating band is not considered to be a full-length translation product. As was mentioned earlier, many less than full-length products are expected during translation of dsRNA.

In part B of Figure 28, the \(^{32}\text{P}\)GMP-labeled bands of RV1 and RV3 (lanes 1 and 4) are compared to the protein profiles (lanes 2 and 3). The protein bands in this part of the figure are not very sharp. However, distortions which are seen in the stained protein lanes (lanes 2 and 3) are mirrored exactly in the autoradiograms (lanes 1 and 4). The distortions confirm the correct alignment of the lanes. In each case, the labeled band comigrated with the second stained band which is lambda 2 as determined in part A of this figure. The location of lambda 3 was determined by staining of overloaded lanes (not
shown). The separation of the lambda proteins, especially the separation between lambda 2 and lambda 3, is improved in this gel system as compared to usual polyacrylamide gels containing acrylamide to bisacrylamide ratios of 29.2 : 0.8 (Fig. 26).

**SDS-PAGE in Gels with Reduced Crosslinking.** The conditions of Laemmli (1970) were used with the following changes. Resolving gels of 6% acrylamide and stacking gels of 3.3% acrylamide in a ratio of 29.6 g acrylamide : 0.4 g bis acrylamide (one-half usual crosslinking) were used. Electrophoresis was for 4 hours at 50 mA. The lambda proteins migrated to within 2 cm of the bottom of the gel under these conditions.

The migration pattern of the lambda proteins on SDS-polyacrylamide gels with one-half the usual amount of crosslinking is shown in Figure 29. Lanes 1 to 5 show RV3-derived proteins. In lanes 3, 4 and 5, arrows indicate the full-length $[^{35}\text{S}]$methionine-labeled translation products of dsRNA segments L1, L2 and L3, namely lambda 3, lambda 2 and lambda 1 respectively. Under these electrophoretic conditions, lambda 2 migrated more slowly than lambda 1 and the separation between lambda 2 and lambda 3 was very much improved. Lanes 1 and 2 represent the autoradiogram and stained protein pattern, respectively, of $[^{32}\text{P}]$CMP-labeled RV3. The $[^{32}\text{P}]$ label comigrated with the slowest migrating protein band which is lambda 2 as determined in lane 4 by translation of the L2 gene. Similar results were obtained using RV1 derived proteins (lanes 6, to 10). In lane 8, the translation products lambda 2 and lambda 1 were both evident. The slowest band comigrated with lambda 2 in lane 7 and could be
attributed to the contamination of RV1 L3 with L2 (see Fig. 23). The results clearly demonstrate that the guanylylated proteins of RV1 and RV3 comigrated with lambda 2 and not with lambda 3. In the two SDS-polyacrylamide gel systems described, the order of migration of lambda 1 and lambda 2 relative to each other is switched. The $[^{32}P]GMP$-labeled band comigrated with lambda 2 in each case. These results argue against a shift in migration of the labeled band as a consequence of the GMP associated with the intermediate.

Summary

The possibility that lambda 1 was the guanylyltransferase was eliminated by analysis of guanylylated proteins on phosphate-urea gels. As well, results of proteolytic treatment of top component and alkali treatment of cores indicated that label was not associated with lambda 1 but was associated with either lambda 2 or lambda 3. Two alternative SDS-polyacrylamide gel systems were developed to improve the resolution of lambda proteins. The genetic origin of the protein bands detected on these gels and on phosphate-urea gels was determined by in vitro translation of the purified dsRNA genome segments. The migration patterns of the lambda proteins were unique in the phosphate-urea and in two of the SDS-polyacrylamide gels. The $[^{32}P]GMP$-labeled protein comigrated with lambda 2 in each case for both RV3 and RV1. Therefore, it is concluded that the RV3 and RV1 L2 gene product, lambda 2, is the reovirus guanylyltransferase.
Figure 19. Chymotrypsin Digestion of Labeled Top Component.

RV3 top component (TC) was incubated with [alpha-32P]GTP and half the sample was further digested with chymotrypsin. Labeled proteins were analyzed by electrophoresis on a 7.5%-polyacrylamide phosphate-urea gel at 50 mA for 22 h.

Lane 1: autoradiogram of the labeled proteins of TC.
Lane 2: autoradiogram of the labeled proteins of TC after chymotryptic digestion of the labeled particle.
Lane 3: Coomassie blue-stained protein profile of lane 1 (TC).
Lane 4: Coomassie blue-stained protein profile of lane 2 (TC+CH).
Figure 20. Analysis of Alkali-Treated Labeled Cores by Polyacrylamide Gel Electrophoresis.

Half of a sample of purified $^{32}$P-GMP-labeled cores were incubated at pH 11.8 according to the method of White and Zweerink (1976). After 15 min at 4°C, cores were sedimented at 165,000 x g for 10 min. The supernatant fraction was neutralized and the pellet washed once. Proteins in each fraction were analyzed by electrophoresis on a 7.5% polyacrylamide-phosphate-urea gel at 50 mA for 22 h. Coomassie blue-stained proteins were compared to the radioactive protein profile of the labeled cores before treatment, the pH 11.8 pellet and the pH 11.8 supernatant (sup't). Coomassie blue-stained RV3 proteins are included as markers.
Figure 21. Analysis of dsRNA Genomic Segments of RV1 and RV3 by Polyacrylamide Gel Electrophoresis.

Total dsRNA from solubilized RV1 and RV3 virions was analyzed by electrophoresis in an SDS-7.5%-polyacrylamide gel at 150 V for 6 h. dsRNA segments were detected by silver staining. Large (L), medium (M) and small (S) size classes are indicated. Lambda proteins, which migrate just ahead of the S2 segment, were also detected by the silver stain.
Figure 22. Analysis of Purified dsRNA L Segments of RV3 by Polyacrylamide Gel Electrophoresis.

The RV3 dsRNA gene segments L1, L2 and L3 were purified according to the method of McCrae and Joklik (1978) by electroelution of the segments out of gel slices. The eluted dsRNA was extracted with phenol and isoamyl alcohol and precipitated in ethanol. The three dsRNA segments and total dsRNA of RV3 were analyzed by electrophoresis in 5%–polyacrylamide–TAE gels at 120 V for 5 h. The segments were detected by ethidium bromide fluorescence under U.V. light.
Figure 23. Analysis of Purified dsRNA L Segments of RV1 by Polyacrylamide Gel Electrophoresis.

The RV1 dsRNA gene segments L1, L2 and L3 were purified according to the method of McCrae and Joklik (1978) by electroelution of the segments out of gel slices. The eluted dsRNA was extracted with phenol and isoamyl alcohol and precipitated in ethanol. The three dsRNA segments and total dsRNA of RV1 were analyzed by electrophoresis on 5%-polyacrylamide-TAE gels at 120 V for 5 h. The segments were detected by ethidium bromide fluorescence under U.V. light.
Figure 24. Dot Blot Hybridization Analysis of RV1 and RV3 dsRNA Segments.

Unlabeled RV3 dsRNA segments L1, L2 and L3 were denatured and baked onto individual nitrocellulose filters (3 filters each, duplicate dots). RV1 dsRNA segments L1, L2 and L3 were labeled at the 3'-termini with [5'-32P]cytidine 3',5'-bis phosphate (pCp) and denatured. Each RV1 segment was hybridized to each RV3 segment at 65°C. The filters were washed, dried and spots detected by autoradiography. Detection of label indicates sequence homology between the RV1 and RV3 segments.
Figure 25. Agarose Gel Analysis of RV3 dsRNA L Segments Under Denaturing Conditions.

Purified RV3 dsRNA segments L1, L2 and L3 were denatured with methyl mercury hydroxide and electrophoresed on a 1.5%-agarose-methyl mercury hydroxide gel at 70 V for 4 h. Markers (number of bases) were Lambda phage DNA digested with the restriction endonuclease Hind III. The gel was soaked in beta-mercaptoethanol and ethidium bromide and the bands visualized by fluorescence under U.V. light.
Figure 26. Analysis of In Vitro Translation Products of RV3 dsRNA L segments by Polyacrylamide Gel Electrophoresis.

Purified RV3 dsRNA segments L1, L2 and L3 were denatured and translated in L-cell translation lysates at 30°C for 1 h according to the method of Skup and Millward (1977). The [35S]methionine-labeled products were solubilized and analyzed by electrophoresis on an SDS-7.5%-polyacrylamide gel at 50 mA for 3 h. Also analyzed were control translations done with no added RNA (-) and with total L-cell mRNA (uninf. mRNA). In vivo [35S]methionine-labeled proteins from a reovirus-infected L-cell lysate (35S S-200) are included as reovirus protein size markers. The largest products of translation of dsRNA segments L1, L2 and L3 are full-length proteins lambda 3, lambda 2 and lambda 1 respectively. Multiple bands migrating as less than full-length represent incomplete translation products due to renannealing of the dsRNA genome strands during translation, or they may arise from aborted internal initiations. Bands were detected by autoradiography.
TRANSLATION

35S
dsRNA
S-200
L1  L2  L3
UNINF.
mRNA

λ
μ
σ
Figure 27. Analysis of Lambda Proteins from RV1 and RV3 by Phosphate-Urea Polyacrylamide Gel Electrophoresis.

The $[^{35}\text{S}]$methionine-labeled in vitro translation products of the RV3 L dsRNA gene segments and the $[^{32}\text{P}]$GMP-labeled proteins of RV1 and RV3 were analyzed by electrophoresis in a 7.5%-polyacrylamide phosphate-urea gel at 50 mA for 22 h. Bands were detected by Coomassie blue staining or by autoradiography.

Part A.
Lane 1: translation products (lambda 1, arrow) of RV3 L3 gene.
Lane 2: translation products (lambda 2, arrow) of RV3 L2 gene.
Lane 3: translation products (lambda 3, arrow) of RV3 L1 gene.
Lane 4: autoradiogram of $[^{32}\text{P}]$GMP-labeled RV3 core proteins.
Lane 5: Coomassie blue-stained RV3 core proteins shown in lane 4.
Lane 6: Coomassie blue-stained RV3 proteins used as size markers.

Part B.
Lanes 1, 3 and 5: Coomassie blue-stained proteins of RV3, RV3 top component and RV1, respectively.
Lanes 2, 4 and 6: Autoradiograms of $[^{32}\text{P}]$GMP-labeled proteins of RV3, RV3 top component and RV1, respectively.
Figure 28. Analysis of Lambda Proteins from RV1 and RV3 on SDS-7.5%-Polyacrylamide Gels with Increased Crosslinking.

The $[^35]S$ methionine-labeled *in vitro* translation products of the RV1 and RV3 dsRNA gene segments and the $[^32]P$GMP-labeled proteins of RV1 and RV3 were analyzed by PAGE at 50 mA for 6 h. The SDS-7.5%-polyacrylamide gel was prepared with increased amounts of crosslinking agent, the acrylamide ratio being 28.4 acrylamide : 1.6 g bis-acrylamide. Bands were detected by Coomassie blue staining or by autoradiography. The top of the gel is not included in the figure since no bands were detected in that region.

Part A.

Lanes 1 to 3: RV1 translation products.
Lanes 4 to 6: RV3 translation products.

Lanes 1 and 4: translation products (lambda 3, arrow) of L1 gene.
Lanes 2 and 5: translation products (lambda 2, arrow) of L2 gene.
Lanes 3 and 6: translation products (lambda 1, arrow) of L3 gene.

Part B.

Lane 1: autoradiogram of $[^32]P$GMP-labeled RV1 proteins.
Lane 2: Coomassie blue-stained RV1 proteins.
Lane 3: Coomassie blue-stained RV3 proteins.
Lane 4: autoradiogram of $[^32]P$GMP-labeled RV3 proteins.
Figure 29. Analysis of Lambda Proteins from RV1 and RV3 on SDS-6%–Polyacrylamide Gels with Reduced Crosslinking.

The [35S]methionine-labeled in vitro translation products of the L dsRNA gene segments and the [32P]GMP-labeled proteins of RV3 (lanes 1 to 5) and RV1 (lanes 6 to 10) were analyzed by PAGE at 50 mA for 4 h. The SDS-6%–polyacrylamide gel was prepared with a reduced amount of crosslinking agent, the ratio of acrylamide being 29.6 g acrylamide : 0.4 g bis acrylamide. Bands were detected by Coomassie blue staining or by autoradiography. The top of the gel is not included in the figure since bands were not detected in that region.

Lanes 1 and 10: autoradiogram of [32P]GMP-labeled virus proteins.

Lanes 2 and 9: Coomassie blue-stained protein profiles of lanes 1 and 10, respectively.

Lanes 3 and 6: translation products (lambda 3, arrows) of L1 gene segments.

Lanes 4 and 7: translation products (lambda 2, arrows) of L2 gene segments.

Lanes 5 and 8: translation products (lambda 1, arrows) of L3 gene segments.
CHAPTER IV

DISCUSSION
The reovirus guanylyltransferase has been identified as the core-associated viral protein lambda 2 (140 kd) by analysis of a $[^{32}\text{P}]\text{GMP}$-labeled covalent reaction intermediate. Like other guanylyltransferases, the reovirus enzyme reacts with GTP to form an enzyme-GMP intermediate. GMP is first linked to the enzyme by a 5'-phosphoamide bond and is then transferred from the enzyme to 5'-ppG-terminated mRNA molecules to form G(5')ppp(5')G cap structures.

**The Guanylyltransferase Reaction**

The guanylyltransferase reaction can be described by a ping pong mechanism:

\[
\begin{align*}
\text{GTP} & \quad \text{PPi} & \quad \text{ppG...} & \quad \text{GpppG...} \\
\downarrow & & \downarrow & \downarrow \\
E & \quad \text{E-pG} & \quad \text{E}
\end{align*}
\]

(1) Guanylylation  (2) Transguanylylation

Reaction 1 in the forward direction is demonstrated by formation of a $[^{32}\text{P}]\text{GMP}$-labeled intermediate upon incubation of enzyme with $[^{\alpha-32}\text{P}]\text{GTP}$. The reaction soon reaches a plateau but in the presence of pyrophosphatase, linear reaction rates can be maintained for extended times. The pyrophosphatase removes PPi from the reaction mixture, thereby promoting the forward reaction. In the absence of pyrophosphatase, the level of accumulated $[^{32}\text{P}]\text{GMP}$-labeled intermediate reaches a plateau, but then begins to decrease. The disappearance of intermediate can be explained by analyzing the products formed at this time in the reaction. Results show that Gp4G cap structures are formed when enzyme-GMP intermediate is incubated with GTP. This indicates that GTP participates not only in guanylylation but also as a substrate for cap formation in vitro.
Therefore, a decrease in detectable intermediate will occur when the formation of capped product exceeds the formation of intermediate.

A previously reported PPi-GTP exchange reaction (Wachsman et al., 1970) can be explained by the reversal of reaction 1, where incubation of cores with cold GTP and \(^{32}\text{P}\)-labeled PPi results in the recovery of labeled GTP. I have also found that incubation of purified \(^{32}\text{P}\)GMP-labeled cores with PPi results in rapid reduction of labeled intermediate with concurrent formation of labeled GTP.

Purified \(^{32}\text{P}\)GMP-labeled cores can be used to demonstrate the second reaction by incubation with GDP to yield unlabeled enzyme and the \(^{32}\text{P}\)-labeled cap structure, GpppG. The reversibility of the second reaction was not investigated but the possibility could be demonstrated by incubation of cores with labeled cap structures to determine whether \(^{32}\text{P}\)GMP-labeled enzyme intermediate is formed. Furuichi et al., (1976) showed that incubation of cores with PPi and labeled GpppGpC resulted in the formation of labeled GTP and ppGpC.

Thus, the reverse reaction can occur but the forward reaction is probably favored in vivo. Immediate methylation in vivo of the nascent cap structures by the virion associated methyltransferases would effectively remove the cap structures from reaction 2, pulling the reaction in the forward direction.

Reactions 1 and 2 were studied concurrently by addition of various nucleotides to a guanylylation reaction that was already in progress. In addition to demonstrating which nucleotides could act as cap acceptors, the results indicate that some of the nucleotides also inhibited the guanylylation reaction. Addition of GMP (not a substrate for transguanylylation) to the guanylylation reaction inhibited formation of intermediate somewhat. GMP may bind to the enzyme and
thus prevent the binding of GTP. Pyrimidine nucleoside diphosphates effectively stopped the formation of labeled intermediate. Products were not analysed, but since pyrimidine termini are not substrates for viral guanylyltransferases in general (Banerjee, 1980), cap formation is probably not a factor in the reduction of intermediate in this case. The inhibition of intermediate formation by pyrimidine nucleoside diphosphates was stronger than that of GMP and could indicate blockage of the active site. In contrast, purine nucleoside di- and tri-phosphates caused a rapid decrease of preformed intermediate. Analysis of the products showed that cap structures were formed.

Therefore, two events may be taking place during these reactions. The nucleotides may reduce further intermediate formation by inhibiting the enzyme or by substituting for labeled GTP, or they may cause a decrease in labeled intermediate via cap formation by reaction 2. The latter is easily verified by monitoring cap formation as was done for the reactions with purine acceptors. The former possibility could be assessed by monitoring formation of intermediate only if there is no cap formation via reaction 2. However, in the case where cap structures were formed on purine acceptors, the purine nucleotides might have also caused a decrease in intermediate by inhibiting reaction 1. To test for this possibility, it would be necessary to monitor the production of labeled PPI. This could be done by analyzing aliquots of the reaction at various times by PEI Cellulose thin layer chromatography. If the purine nucleotides are only acting as cap acceptors, then the level of PPI would continue to increase even as
intermediate disappears. If the purine nucleotides are also inhibiting reaction 1, then the rate of PPi production would be reduced depending on the inhibitory effect of the nucleotides. In the case where the purine nucleotide was GTP, the level of detectable \(^{32}\text{P}\)GMP-labeled intermediate would also decrease simply due to dilution of labeled GTP with unlabeled GTP in the guanylylation reaction but the rate of production of PPi would be unchanged.

The active site of the enzyme must contain 2 binding subsites, one for GTP and the other for the 5'-ppG terminus of nascent mRNA. Removal of PPi from subsite 1 after GTP binding may be necessary for subsequent binding of nascent mRNA in subsite 2. The enzyme could then catalyze the condensation of the two molecules to yield capped mRNA. Chemical crosslinking studies using oxidized nucleotides might be useful in clarifying the events occurring in the active site.

**Lambda Cleavage Product**

The guanylyltransferase of cores, made in vitro by chymotrypsin digestion of virions, exists in the form of lambda 2 as well as a polypeptide that is smaller than lambda 2 by about 15-20 kd. Peptide map analysis indicated that these polypeptides are related and the smaller one was denoted lambda 2C, for "cleavage product of lambda 2". PaSVPs do not contain detectable lambda 2C but the cleavage product could be labeled with GMP after chymotrypsin digestion of PaSVPs. Therefore, it was concluded that the lambda 2C of cores must also have been formed by chymotryptic cleavage of lambda 2.

A lambda cleavage product with an electrophoretic mobility similar to lambda 2C has been noted by Lee et al., (1981a), Samuel, (1983) and
Shatkin et al. (1983). In the latter report, the cleavage product was detected as a GMP-binding protein which was also called lambda 2C although the identities of the $[^{32}\text{P}]$GMP-labeled lambda and lambda C bands were not rigorously established. Their relationship was not established by peptide mapping but was surmised since both proteins bound GMP. The lambda cleavage product described by Lee et al. was detected as a soluble protein in infected cell lysates and was determined to be derived from lambda 2 by immunoprecipitation with anti-lambda 2 monoclonal antibody. I attempted to use anti-lambda 2 monoclonal and anti-lambda 1 monospecific antibodies, obtained from Dr. Patrick Lee, Calgary, Alberta, to identify the $[^{32}\text{P}]$GMP-labeled lambda protein in my studies. The results were inconclusive, perhaps due to the SDS required to solubilize the viral proteins from labeled viral particles. A lambda cleavage product was also detected in in vivo $[^{35}\text{S}]$methionine-labeled infected cell lysates during the course of my work. The electrophoretic mobility of the lysate polypeptide in an SDS-polyacrylamide gel was slightly faster than the $[^{32}\text{P}]$GMP-labeled lambda 2C core polypeptide, possibly due to the GMP group on the latter. Lambda 2C was not detected as a $[^{32}\text{P}]$GMP-binding protein in the S-200 fractions of infected L-cells (Chapter III, Section 1) but may nevertheless have been present as an inactive polypeptide. Thus, it remains possible that the lambda cleavage product of infected cell lysates is different from the core-associated lambda 2C that binds GMP. The difference may reflect an alternative cleavage site of lambda 2 which renders the in vivo lambda 2C inactive with respect to binding of GMP.
A lambda cleavage product described by Samuel was detected both in infected cell lysates and in virions. The polypeptide was not characterized. During my study, lambda 2C was never detected in virions that were freshly-prepared and only frozen once (Chapter III, Section 3). However, some old stocks of virus kept only for use as marker proteins had variable amounts of a lambda cleavage product as seen by Coomassie blue protein staining. The repeated freeze-thawing of the samples may have accelerated degradation of lambda 2.

Nevertheless, the lambda 2C that was formed by chymotrypsin digestion of paSVPs was an active GMP-binding protein and comigrated with the labeled lambda 2C of cores. Although lambda 2C appears to be present in the cytoplasm of infected cells as a polypeptide which is inactive in GMP binding, it was not detected in native parental SVPs and must not be particle-associated at least at early times post infection.

The identity of the [32P]GMP-labeled peptide maps of lambda 2 and lambda 2C and the ability of lambda 2C to function effectively as an enzyme suggest that the chymotrypsin cleavage site is structurally and functionally removed from the GMP-binding site. It might have been expected that chymotrypsin cleavage of lambda 2 would result in reduced enzyme activity. However, PAGE analyses routinely show that [32P] label in the lambda 2C band is equal to or greater than that in the lambda 2 band while the amount of protein in the lambda 2C band is less than one-fifth that of lambda 2. It is possible that chymotrypsin cleavage improves accessibility of GTP to the enzyme. Conversely, chymotrypsin cleavage of lambda 2 may yield a molecule with a higher affinity for GTP by altering the conformation of the
active site. However, calculations indicate that under the conditions of labeling described (2.5 h, 20°C), only 0.2 molecules of GMP are bound per core or approximately 1 GMP per 300 lambda 2 molecules. Morgan and Kingsbury (1980) showed that pyridoxal phosphate interacts with only 10% of lambda 1 and 10% of lambda 2/lambda 3 and that this amount of interaction was sufficient to inactivate transcription. The interaction with 10% of lambda 2 might have actually corresponded to interaction with all of the lambda 3 molecules. Nevertheless, they proposed that most of the lambda 1 and lambda 2 proteins probably had a structural role only. If this is true, then it would partly explain the low level of GMP-binding that I have observed.

Cap Formation on Reovirus Messenger RNA

Full-length 5'-ppG-terminated reovirus mRNA could not be capped in the in vitro transguanylylation reaction. Normally capping occurs quite early during viral RNA synthesis, probably at the dinucleotide level (Furuichi et al., 1976). These early reactions must take place within the viral core where the template dsRNA segments of the viral genome reside. Thus, the inability of the guanylyltransferase to cap exogenous mRNA may be due to the inability of large RNA molecules to diffuse into the core particle. Possible interaction of the 3'-terminus with the 5'-terminus of full-length reovirus mRNA may also interfere with cap formation on completed molecules (Antczak et al., 1982). Consistent with this interpretation is the ability of cores to cap oligonucleotides produced by abortive RNA synthesis. These molecules appear to be small enough to enter the core and approach the guanylyltransferase active site. Shatkin et al., (1983) reported that
they could not demonstrate cap formation by the guanylyltransferase intermediate. According to their report, it appears that they were using the guanylyltransferase intermediate of virions instead of cores. The proposed structure of virions is such that mRNA cannot escape from the spike after assembly of the outer capsid proteins. The structural block may be partly responsible for the synthesis of oligonucleotides as abortive transcripts. Since the oligonucleotides are released from the virion only after removal of the outer capsid, the converse could also be assumed, namely that exogenous mRNA molecules would be unable to enter the core when the spikes are blocked by outer capsid proteins. Shatkin's group attempted to use both full-length mRNA and 5'-ppGpC as cap acceptors in their experiments suggesting that even dinucleotides cannot approach the guanylyltransferase when the outer capsid proteins are present.

**Functional Differences Between Parental SVPs and Cores**

Although paSVPs and cores both exhibit active guanylyltransferase, some differences do exist with regard to substrate specificity. The core-associated enzyme can cap 5'-ppG and 5'-ppA terminated oligonucleotides whereas the paSVP-associated enzyme can cap 5'-ppG termini only. After the transfer reaction, label is lost from both lambda 2 and lambda 2C in cores, demonstrating that lambda 2C is active in transfer as well as binding of GMP. The broader substrate specificity of cores may in part be attributable to the presence of lambda 2C. Other polypeptide differences also exist between cores and parental SVPs, notably the presence in the latter of the mu 1C cleavage product, delta. A test for the role of lambda 2C in substrate
specificity would be to use only 5'-ppA-terminated oligonucleotides in
the transguanylylation reaction and determine if label is lost only
from lambda 2C intermediate. If this is not the case, cleavage of
lambda 2 or the presence of the mu 1C cleavage product, delta, may
have affected the overall conformation of the core, causing lambda 2
and lambda 2C both to be less specific with respect to cap acceptor
substrate.

The biological significance of the inability of paSVPs to cap
oligoadenylates is not apparent although a similar specificity was
noted when virions were used to study methylation of oligomers in
vitro (Carter, 1977). Only 5'-G-terminated oligonucleotides could be
methylated. Other work has indicated that viral protein mu 1C may be
modified by covalent addition of oligo(A) linked through the
3'-terminus (Carter et al., 1980). If the reovirus oligoadenylates are
the source of the oligo(A) bound to mu 1C, it may be important that
the unattached 5'-terminus remain uncapped.

On the other hand, the ability of paSVPs to cap 5'-ppG-terminated
oligonucleotides may be important during infection. When virions are
uncoated in vivo, there is an immediate release of thousands of
oligonucleotides into the cytoplasm of the cell. These molecules,
which can become capped upon release (Carter and Lin, 1979), might
interact with cap binding proteins and other initiation factors in the
cytoplasm. Cap-dependent translation would be reduced in the same way
that m7GDP cap analogue inhibits translation of capped mRNA in
vitro. This would assist in the translational takeover of the cell by
reovirus although initial translation of capped reovirus mRNA would
also be decreased.
Tetraphosphate Cap Structure

Cores catalyze the formation of the cap-like structure, diguanosine tetraphosphate, when GTP is used as the cap acceptor in transguanylylation (Chapter III, Section 2). Structures of this type have been described in other systems, including yeast (Wang and Shatkin, 1984). The purified yeast guanylyltransferase, free of phosphohydrolase activity, could form tetraphosphate cap structures using GTP as a cap acceptor. GpppN structures were also formed during in vitro transcription catalyzed by another member of the Reoviridae, cytoplasmic polyhedrosis virus (Smith and Furuichi, 1982), and GpppG has been used to prime nascent transcription in reovirus (Yamakawa et al., 1982a). GpppG could not be used in the same manner to prime transcription whereas the transcripts that contained the tetraphosphate primer could not be methylated. Human reovirus is unable to cap mRNA with 5'-gamma-S-GTP termini during in vitro transcription (Reeve et al., 1982), suggesting that transcription-coupled cap formation requires prior removal of the 5'-terminal gamma phosphate. These results suggest that formation of GpppG does not occur during normal transcription-coupled mRNA modification but rather may be an artifact of in vitro transguanylylation. I have found that when cores or paSVPs are incubated with reovirus oligonucleotides, only cap structures with triphosphate bridges are formed. Since the population of oligonucleotides is known to contain triphosphate-terminated molecules, it may be that the enzyme preferentially uses 5'-diphosphate termini when given the choice. In other words, the
affinity of the enzyme for 5'-diphosphate-terminated acceptors may be higher than for 5'-triphosphate-terminated acceptors. Another explanation may be that the core-associated phosphohydrolase removes the gamma phosphate of oligonucleotides before capping. It is not known why the phosphohydrolase did not also remove the gamma phosphate of GTP to prevent formation of GppppG cap structures. The original demonstration of the phosphohydrolase activity involved measuring hydrolysis of ATP and GTP as well as CTP and UTP to a lesser extent (Borsa et al., 1970). Since the gamma phosphate of GTP was not removed before capping, it may be that the guanylyltransferase reaction is faster than the phosphohydrolase reaction. Perhaps oligonucleotides, as more authentic analogues of nascent mRNA, cannot evade the phosphohydrolase activity as did GTP. The 5'-termini of the oligonucleotides used in my studies were not examined. It remains possible that the gamma phosphates were removed during isolation from chymotrypsin-treated virions. As virions were being converted into cores, the oligonucleotides may have been acted on by the phosphohydrolase before release from the particles.

Soluble Enzyme

Reports have been made on the isolation of soluble reovirus enzyme activities. These include the solubilization of a transcriptase activity (Comatos, 1968) and an ATPase (Banerjee, 1981). However, careful analysis of the experimental conditions of those studies indicates that the data did not support the existence of a soluble enzyme. The transcriptase activity reported by Comatos (1968) was identified in crude fractions which would have contained viral
particles. The ATPase activity was studied by measuring the presence of unlabeled inorganic phosphate in the post-core supernate after addition of ATP. The ATPase activity was only detected in soluble form if cores were incubated with ATP before centrifugation. The liberated phosphate was therefore derived from core-associated enzyme activities which hydrolyzed ATP during preincubation and hence the data do not support the presence of a soluble ATPase.

Several reovirus proteins with non-enzymatic binding properties have been purified. Reovirus proteins sigma 3 and sigma NS were purified by affinity chromatography based on their nucleic acid binding properties (Huismans and Joklik, 1976). Purified sigma 3 can be used to stimulate in vitro translation of uncapped reovirus mRNA in uninfected L-cell lysates (Lemieux et al., submitted). Expression of a cloned S3 gene in E. coli yields a sigma NS protein which binds to ssRNA like the authentic protein (Richardson and Furuichi, 1985). A sigma 1-lac Z fusion protein made by expression of the S1 gene in E. coli, has hemagglutination activity and can compete with reovirus for L-cell surface receptors (Masri et al., 1986). Aside from these binding activities, solubilization or purification of reovirus enzymes in an active form has not been demonstrated.

During the course of this study, I made several attempts to solubilize the reovirus guanylyltransferase. The soluble fractions of uninfected and reovirus-infected L-cell lysates were tested for the presence of a GMP-binding protein by incubation with [alpha-32P]GTP. A [32P]-labeled band comigrating with the reovirus lambda proteins was detected only in reovirus-infected cell lysates and may represent
the GMP-binding activity of the reovirus guanylyltransferase. However, it is possible that the activity was due to some residual viral particles that were not completely cleared from the soluble fraction by differential centrifugation. Attempts to label lambda 2 that had been removed from cores by incubation at pH 11.8 (Chapter III, Section 3, Alkali Treatment of Cores), by incubation with [alpha-$^{32}$P]GTP, were unsuccessful. Immunoprecipitated, SDS-solubilized lambda 2 also did not bind GMP either before or after removal of the protein from the antibody. Incubation at high pH and in SDS are both relatively harsh conditions. To circumvent the need to solubilize the protein from particles, lambda 2 was synthesized in vitro by translation of the purified dsRNA L2 gene segment (Chapter III, Section 3). However, the translation product could not be labeled with $[^{32}$P]GMP even after removal from the translation lysate by immunoprecipitation. Although it was possible that the antibodies were preventing interaction of GTP with the soluble protein, the antibodies did not inhibit GMP binding to lambda 2 in reovirus cores. Further attempts to solubilize the guanylyltransferase could involve isolation of the in vitro translation product, lambda 2, on a GTP affinity column. Binding to the column would, itself, be a measure of GMP binding activity of the enzyme.

Identification of the Guanylyltransferase Protein

Identification of the protein with the guanylyltransferase activity proved to be the most difficult aspect of this work. The main reason for this was the lack of a good system for separation of the reovirus lambda proteins. The currently used conditions of PAGE
include the SDS-polyacrylamide gel system described by Laemmli (1970) and the phosphate-urea gel system. Both are inadequate for analysis of reovirus lambda proteins. Separation of the lambda proteins on SDS-polyacrylamide gels is very poor and I have found that protein concentration can affect the mobility of this group of proteins. In addition, the poor resolution of the lambda proteins lends credence to the argument that a GMP ligand may slightly alter the mobility of the labeled protein with respect to the unlabeled homologous protein. In phosphate-urea gels, the separation of lambda 1 from lambda 2 and lambda 3 is good but the latter two proteins migrate as one band. The mobility of proteins in this system is largely independent of protein concentration. Using phosphate-urea PAGE and experimentally altered \[^{32}\text{P}]\text{GMP}-labeled viral particles, it was possible to exclude lambda 1 as possessing the guanylyltransferase activity. Further distinction between lambda 2 and lambda 3 required use of the SDS-polyacrylamide gel system of Laemmli (1970). First, it was necessary to develop conditions of electrophoresis that would better resolve the lambda proteins. Manipulation of the composition of SDS-polyacrylamide gels demonstrated that reducing or increasing the polymer crosslinking of the gel greatly improved resolution of the lambda proteins. The identities of the protein bands obtained under the new PAGE conditions were determined by their comigration with full-length in vitro translation products of the individual purified large dsRNA segments. The results indicated that the order of migration of the three lambda proteins under these conditions could not be assumed merely on the basis of electrophoretic mobility and
apparent molecular weight in other electrophoresis systems. The order in gels with increased crosslinking was the same as had been determined by McCrae and Joklik (1978), lambda 1, lambda 2 and lambda 3 (slowest to fastest). In contrast, in gels with one-half the usual crosslinking, the lambda proteins migrated in the order lambda 2, lambda 1 and lambda 3 (slowest to fastest). In addition to the greatly improved resolution of all three lambda proteins in both types of gels, the switch in migration pattern of the lambda proteins was useful in identifying the guanylyltransferase. The $^{32P}$GMP-labeled band comigrated with lambda 2 in each case. Under these circumstances, it is difficult to argue that the presence of GMP on either lambda 1 or lambda 3 cause them to migrate in the position of lambda 2 in all four gel systems used. The results indicate that GMP was indeed bound to lambda 2. The results also demonstrate the necessity of re-establishing the identity of protein bands whenever electrophoretic conditions are changed.

**The Core-Associated Enzyme Complex**

I have shown here that core protein lambda 2 is the reovirus guanylyltransferase. Using genetic reassortment experiments, Drayna and Fields (1982a) have provided evidence that the viral transcriptase activity is associated with lambda 3. Still awaiting assignment to specific polypeptides are the core-associated methyltransferase and phosphohydrolase activities. Morgan and Kingsbury (1980, 1981) have shown that pyridoxal phosphate binds to both lambda 1 and lambda 2 (lambda 3) and can interact with the S-adenosyl-methionine binding site(s) of the methyltransferase. The results suggest that one or both
methyltransferase activities are associated with one or more of the lambda proteins. Thus, lambda 1 may be a methyltransferase, or lambda 2 or lambda 3 may be bifunctional proteins containing a methyltransferase activity. I made preliminary attempts to identify the reovirus methyltransferase by formation of a $[^{3}H\text{-methyl}]$-S-adenosylmethionine reaction intermediate but could not detect any labeled proteins. Nothing is known about the identity of the phosphohydrolase. A recent study by Morgan and Kingsbury (1983) showed that GTP can be crosslinked to cores by U. V. irradiation. The GTP was linked to lambda 2 (lambda 3) as determined by phosphate-urea PAGE. All four NTPs could be crosslinked to a lesser extent to lambda 2 (lambda 3) and to sigma 2. With lambda 2 as the guanylytransferase, crosslinking of NTPs to lambda 3 correlates with its possible role as the transcriptase. Crosslinking of NTPs to sigma 2 raises the possibility that it also plays a role in transcription, possibly as a phosphohydrolase. Sigma 2 can be iodinated in cores but to a lesser extent than lambda 2 or even lambda 1 (White and Zweerink, 1976) suggesting that sigma 2 is located more internally than lambda 1 in the core.

Spike Structure and Function

The mRNA synthesis and modification enzymes of reovirus appear to be closely associated and dependent on the structural integrity of the core since they cannot be purified in an active form. Other examples of enzyme complexes are the mRNA modifying enzymes of vaccinia virus which have been purified as a multifunctional enzyme complex containing two major polypeptides (Martin et al., 1975; Shuman et al.,...
1980; Venkatesan et al., 1980a), and the guanylyltransferase of yeast which has an associated 5'-triphosphatase in a 4-subunit protein complex (Itoh et al., 1984a). There are also examples of bifunctional enzymes, such as the guanylyltransferase and 5'-triphosphatase activities of rat liver nuclei (Yagi et al., 1983) and Artemia salina (Yagi et al., 1984), both of which reside on a single protein.

Analysis of virion structure by electron microscopy suggests that the spikes are hollow structures (Luftig et al., 1972) through which nascent mRNA is extruded (Bartlett et al., 1974). As mRNA is channeled though the spike, it may be sequentially modified by spatially organized enzymes. The transcriptase would have to be located at the innermost aspect of the core, in close proximity to the dsRNA genome it requires as a template. Polymerization of 2 nucleotides might bring the 5'-pppG terminus near the phosphohydrolase (sigma 2?) for removal of the gamma phosphate. The diphosphate terminus could then be capped by the guanylyltransferase, lambda 2. Lambda 2, which makes up the spike, is probably the outermost protein of this functional unit. Unless lambda 2 also contains the methyltransferase activities, the cap structure would have to fold back in order to be methylated. The flexibility of the triphosphate bridge structure could allow for this folding (Kim and Sarma, 1977). One possibility is that methylation is catalyzed by lambda 1. Iodination studies show that lambda 1 can be labeled after removal of the spikes (White and Zweerink, 1976), suggesting that it is located beneath lambda 2. Evidence from electron microscopic studies suggests that the cap structure may remain attached to cores while the remainder of the mRNA is polymerized and
extruded from the spikes as loops (Bartlett et al., 1974). As the 3'-terminus is completed, possible 5'- and 3'-terminal interaction (Antczak et al., 1982) may allow for release of the completed mRNA molecule. Validation of this theoretical scheme of reovirus mRNA synthesis requires further assignment of enzyme activities to core proteins.

**Progeny Subviral Particles**

At late times during infection of L-cells with reovirus, production of new virions begins with the assembly of progeny subviral particles (prSVPs). These particles resemble cores in that they lack the outer capsid layer of proteins, and they synthesize 90% of the mRNA present in the infected cell. However, prSVPs are active only in the synthesis of uncapped mRNA (Zarbl et al., 1980b). The prSVP preparations used in the studies of Zarbl et al. (1980b) were not pure but represented the slow sedimenting fraction of a glycerol gradient analysis of a reovirus-infected L-cell lysate. The particles were recovered as a pellet and probably contained other viral particles. An improved method for purification of the prSVP is required to determine the absolute enzyme and protein components of the particle and to facilitate in vitro studies on prSVPs. Nonetheless, Morgan and Zweerink (1975) have demonstrated that prSVPs purified from reovirus-infected cells at late times post-infection lack spikes and have reduced amounts of lambda 2 as determined by phosphate-urea PAGE. Identification of lambda 2 as the guanylyltransferase therefore provides an explanation for the absence of capping activities in prSVPs. However, lack of capping due to lack of guanylyltransferase
would result in an mRNA molecule with a 5'-ppG terminus, whereas mRNA synthesized by prSVP preparations has a 5'-pG terminus. Thus an additional activity would be required to remove the beta-phosphate of nascent mRNA. Skup and Millward showed that the beta-phosphate of 5'-ppG-terminated mRNA was selectively removed in infected lysates while the rest of the mRNA molecule was stable (Skup and Millward, 1980a). Therefore, it was postulated that a nucleotide pyrophosphatase activity is associated either with prSVPs or with reovirus-infected L-cell lysates. The enzyme may be either a viral protein or a virally-induced cellular protein. The presence of a pyrophosphatase would further ensure that the uncapped mRNA is not capped by cellular guanylyltransferases which may be present in the cytoplasm.

The absence of the guanylyltransferase in prSVPs and presence of a pyrophosphatase activity would together provide a simple molecular basis for the production of uncapped mRNA's, which play a key role in the ability of reovirus to usurp the host translational apparatus.

Prospectives

Many questions concerning the structure and function of the reovirus particle remain to be answered. Is the methylase activity also absent in prSVPs or is its inactivity merely due to lack of a cap substrate? A test for this possibility would be to investigate the ability of prSVPs to methylate exogenous GpppG cap structures. Why do prSVPs not contain lambda 2 spikes when lambda 2 is certainly in the cytoplasm? Is there a pyrophosphatase protein that binds to prSVPs and thereby temporarily prevents assembly of the spike and if so, how is it removed prior to maturation of the particle? The pyrophosphatase
activity may, for example, be associated with mu NS which is transiently bound to prSVPs (Morgan and Zweerink, 1975). Prior assembly of the spike structure may be necessary for the assembly of the outer capsid proteins. A clue to the role of lambda 2 in virus assembly was obtained by the study of mutants in the L2 gene segment. A temperature sensitive mutant, ts B, is defective in the L2 gene and has been shown to accumulate core-like particles during infection at the nonpermissive temperature, 39°C (Fields et al., 1971). The ts B mutants are dsRNA+, meaning that they must be able to synthesize and package capped reovirus mRNA and replicate it within the core-like particles. This suggests that the ts lesion in the L2 gene does not affect the capping activity of lambda 2 even at the nonpermissive temperature. These particles were further shown to have reduced levels of lambda 2 and contained mu NS as well as other noncore proteins (Morgan and Zweerink, 1974). Therefore, the absence of spikes or the presence of conformationally altered spikes in these particles appears to prevent further maturation into virions. Parental SVPs, on the other hand, contain lambda 2 and are reassembled into mature virions by addition of outer capsid proteins (Silverstein et al., 1970).

Examination of intertypic recombinants during serial passage at high multiplicity of infection was carried out by Brown et al., (1983). The results demonstrate that establishment of a persistently infected state and accumulation of deletion mutants segregated with the reovirus type 3 L2 gene. Under these conditions of passage, the L2 gene may become defective and the lambda 2 protein may be unable to properly cap nascent mRNA. The inability to synthesize capped mRNA may
slow down the viral replicative cycle and result in an apparent persistently-infected stage. Cap structures may also provide the recognition signal for packaging of parental mRNA into nascent replicase particles. The inability of a defective enzyme to correctly and efficiently cap parental transcripts may result in nascent progeny particles with an incomplete complement of genome segments or even completely empty particles.

Reovirus particle reconstitution studies using in vitro synthesized proteins would certainly be useful in clarifying the role of reovirus proteins in virus structure and function. In this respect, the advent of molecular cloning techniques will surely be a boon to reovirus research.
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Original Contributions to Knowledge

1. Direct labeling of the reovirus guanylyltransferase with $^{32}\text{P}\text{GMP}$ has shown the activity to be associated with the lambda 2 protein of reovirus type 1 and reovirus type 3. This represents the first unequivocal assignment of a reovirus enzyme activity to a known reovirus protein.

2. It is demonstrated that the reovirus guanylyltransferase catalyzes the formation of cap structures by transfer of GMP from GTP to the 5'-ppG terminus of nascent mRNA. The reaction occurs via a covalent, 5'-phosphoamide-linked enzyme-GMP intermediate. The intermediate can be detected in virions, parental subviral particles and cores (prepared by chymotryptic digestion of virions in vitro).

3. Parental subviral particles isolated from infected cells catalyze cap formation on 5'-ppG-terminated oligonucleotides only, whereas cores catalyze cap formation on purine nucleoside di- and triphosphate-terminated molecules. Cores also catalyze formation of tetraphosphate cap structures using GTP as a cap acceptor.

4. The guanylyltransferase is also present in cores as a chymotryptic cleavage product of lambda 2, called lambda 2C, which is active in binding of GMP from GTP as well as transfer of GMP to form cap structures.
5. Identification of the reovirus guanylyltransferase was facilitated by improving the separation of the reovirus lambda proteins by altering the SDS-polyacrylamide gel electrophoresis conditions of Laemmli (1970). The order of migration of the three lambda proteins of reovirus types 1 and 3 was determined in the altered SDS-polyacrylamide gel systems, and the order of migration of the three lambda proteins of reovirus type 3 was determined for phosphate-urea polyacrylamide gels. The coding assignments of the reovirus type 1 L gene segments was determined by in vitro translation of the purified dsRNA L gene segments in the presence of methyl mercury hydroxide.

6. Identification of the reovirus guanylyltransferase protein as lambda 2 has provided an explanation for the previously determined lack of capping activity associated with progeny subviral particles.

*This section is a mandatory requirement of Ph. D. theses submitted to the Faculty of Graduate Studies and Research, McGill University, Montreal.*