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Characterization of the Rotavirus NSP5 Hyperphosphorylation and its Interactions with Other Viral Proteins

Thesis Submitted for the Degree of

Doctor Philosophiae

Cadidate:

Ivka Petrova Afrikanova

Supervisor:

Dr. Oscar Burrone

Academic Year 1996/97

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CONTENTS

List of abbreviations

CHAPTER 1

INTRODUCTION

1. Classification 1

2. Structure of rotavirus 3

3. Genome structure 8

4. Rotavirus proteins-structure and function 12

4.1 VP1 12

4.2 VP2 13

4.3 VP3 14

4.4 VP4 14

4.5 VP6 19

4.6 VP7 23

4.7 NSP1 29

4.8 NSP2 30

4.9 NSP3 31

4.10 NSP4 33

4.11 NSP5 35

5. Rotavirus replication cycle 39

5.1 Overview of the replication cycle 39

5.2 Stages of the replication cycle 40

5.2.1 Adsorption, Penetration, and Uncoating 40

5.2.2 Transcription and Replication 42

5.2.3 Assembly 46

5.2.4 Virus release 47

6. General features of protein phosphorylation and O-glycosylation 48

6.1 Substrate specificity of protein kinases 49

6.2 Autophosphorylation 51

6.3 O-glycosylation and phosphorylation reciprocity 51

6.3.1 O-GlcNAc protein modification 51

6.3.2 O-GlcNAcylation sites are similar or identical to kinase sites 52

6.3.3 O-GlcNAc is a regulatory modification that can be dynamically reciprocal with phosphoryaltion 53

7. Significance of the phosphorylation of viral proteins 54

7.1 Occurence 54

7.2 Functional properties of viral phosphoproteins 54

7.2.1 Assembly of the virions 54

7.2.2 The role of viral phosphoproteins in viral regulatory processes 56

8. Protein kinases encoded by viruses 58

8.1 Bacteriophage T7 protein kinase 58

8.2 Protein kinases transduced by retroviruses 59

8.3 Alphaherpesvirus $U_S$protein kinase 61

8.4 Herpesvirus U_L protein kinase 63

8.5 Channel catfish virus protein kinases 64

8.6 Poxvirus and related protein kinases 64

8.7 Baculovirus protein kinase 66

8.8 Protein kinase activity associated with HSV ribonucleotide reductase 66

8.9 Protein kinase activity of hepatitis B virus Hbx protein 67

8.10 Protein kinase activity of VSV L-proteins 68

CHAPTER 2

METHODS

1. Cells and viruses 70

2. Liposome mediated transient transfections of adherent MA104 cells 70

3. Western immunoblot analysis 71

4. Radiolabeling of rotavirus-infected cells with [^{35}S]methionine, [^{32}P]P_i and (1,6)-[^{3}H]glucosamine 72

5. Chemical crosslinking of rotavirus infected and mock infected cells 73

6. UV-crosslinking of rotavirus infected or mock infected cells 73

7. Immunoprecipitations and PAGE analysis 74

8. Sequential immunoprecipitation 74

9. Phosphatase treatments 75

10. *In vitro* phosphorylation 75

11. Phosphoamino acids analysis 76

12. *In vitro* translation 77

13. Production of anti-NSP2 and anti-NSP5 serum 77

14. Oligonucleotide primers 78

15. PCR deletion mutagenesis of NSP5 78

16. Cloning and sequencing of the PCR deletion mutants 80

CHAPTER 3

RESULTS

1. Phosphorylation of rotavirus NSP5 81

1.1 Multiple forms of post-translationally modified NSP5 81

1.2 *In vitro* phosphorylation of NSP5 84

1.3 Analysis of phosphorylated residues 87

1.4 NSP5 phosphorylation in the absence of viral infection 87

1.5 Post-translational modification of *in vitro* translated NSP5 90

1.6 NSP5 O-linked glycosylation and phosphorylation 90

2. Interaction of the rotavirus NSP5 with other proteins 94

2.1 *In vivo* crosslinking of MA104 rotavirus infected cells 94

2.2 Sequential immunoprecipation of crosslinked and non-crosslinked extracts 100

3. Rotavirus NSP2 upregulates the hyperphosphorylation activity of NSP5 *in vivo* and *in vitro* 102

3.1 *In vitro* phosphorylation of NSP5 in the presence of NSP2 102

3.2 Upregulatory role of NSP2 in the NSP5 phosphorylation 106

4. Biochemical characterization of deletion mutants of NSP5 108

4.1 Description of the NSP5 deletion mutants 108

4.2 Expression of NSP5 deletion mutants 112

4.3 Role of NSP2 on the phosphorylation properties of the deletion mutants 113

4.4 Cotransfection of NSP5 mutants 116

CHAPTER 4

DISCUSSION 118

SUMMARY 128

BIBLIOGRAPHY 131

List of abbreviations

ATP deoxy adenosine triphosphate

BSA bovine serum albumin

CIP calf intestinal phosphatase

DNA deoxyribonucleic acid

DOC deoxycholic acid

DTT dithiothreitol

DSP dithiobis(succinimidylpropionate)

EDTA ethylene diamine tetra acetate disodium salt

h hour

kb kilobase

kDa (K) kilodaltot

ER endoplasmic reticulum

MW molecular weight

mRNA messenger ribonucleic acid

NP-40 Nonidet P-40

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffer saline

PCR polymerase chain reaction

RNA ribonucleic acid

SDS sodium dodecyl sulphate

Tris tris (hydroxylmethyl) aminomethane

UV ultraviolet

VP viral protein

NSP nonstructural protein

Rotaviruses were initially characterized as agents associated with gastroenteritis in animals. Latter on it was found that rotaviruses caused diarreal disease primarily in the young, but infection and disease in older children and adults are also common. Rotaviruses have been reported in association with syndromes other than diarrhea (e.g., exanthem subitum, otitic media, necrotizing enterocolitis, liver abscesses). However, at this time no convincing evidence proves that rotaviruses cause any of these syndromes.

Studies since 1973 on the biochemistry, biology, and molecular and antigenic properties of the rotaviruses have resulted in a fairly detailed understunding of virus structure and the lytic cycle of replication. Recognition that four of the five rotavirus nonstructural proteins bind nucleic acids has resulted in the realization that these proteins have unique properties, and the effort to understand their function will provide new fundamental information about RNA synthesis and expression.

This thesis is devoted to the understanding of the function and the biochemical properties of the rotavirus nonstructural protein 5 (NSP5) which is localized in the virosomes-structures where rotavirus replication occurs in the cytoplasm of the infected cells.

CHAPTER 1

INTRODUCTION

1. CLASSIFICATION.

Rotaviruses are classified as a genus in the family Reoviridae which contains eight other distinct genera (1). The unifying features of the rotaviruses that are responsible for their classification into a separate genus are as follows:

a. Mature virus particles are approximately 75 nm (750Å) in diameter and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and a inner core layer.

b. Sixty spikes extend 120Å from the smooth surface of the outer shell.

c. Particles contain an RNA-dependent RNA polymerase and other enzymes capable of producing capped RNA transcripts.

d. The virus genome contains 11 segments of double-stranded RNA (dsRNA).

e. The viruses are capable of genetic reassortment.

f. Virus replication occurs in the cytoplasm of infected cells.

g. Virus cultivation *in vitro* is facilitated by treatment with proteolytic enzymes, which enhances infectivity by cleavage of an outer capsid spike polypeptide.

h. The viruses exhibit a unique morphogenic pathway (i.e., virus particles are formed by budding into the ER and enveloped particles are evident transiently at this stage of morphogenesis; mature particles are nonenveloped and these virions are liberated from infected cells by cell lysis).

Rotaviruses are classified serologically by a scheme that allows for the presence of multiple groups (serogroups) and for the existence of multiple serotypes within each group. Rotaviruses comprise six distinct groups (A through F). Group A, B, and C rotaviruses are those currently found in both human and animals, whereas viruses in groups D, E, and F have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but ressortment does not occur among viruses in different groups (2). The group antigenic determinants (or common antigens) are found in most (if not all) of the structural proteins and probably on many of the nonstructural proteins as well. This has been documented by showing that monospecific antisera and some monoclonal antibodies (MAbs) specific for individual polypeptides cross-react with strains in addition to those to which they were made. Crossreactive epitopes on the inner capsid protein (VP6) are those usually detected in diagnostic ELISA (3). The common antigen, detected as the cross-reactive antigen among all group A rotaviruses, has been shown to segregate with genome segment 6 and VP6 in reassortment experiments.

Group A rotaviruses have clearly been established as causing significant diarrheal disease in the young. Group B rotaviruses have been assosiated with animal epidemics of severe diarrhea, primarily in adults in China (4, 5, 6). Group C viruses have been sporadically reported in fecal specimens from children with diarrhea; their clinical significance remains unclear.

Within each group, rotaviruses are classified into serotypes defined by reactivity of viruses in plaque reduction neutralization assay using hyperimmune serum prepared in antibody-negative animals (7, 8). Using such assays, 14 VP7 (G) serotypes have been identified and strains of animal and human origin may fall within the same serotype (G serotype because VP7 is a glycoprotein). Neutralization assays can measure reactivity of antibody with the two outer capsid-neutralizing antigens (VP4 and VP7). However, in most cases the predominant reactivity is directed against the glycoprotein

VP7. This may be because VP7 comprises a greater percentage of the virion outer capsid, or alternatively because with hyperimmunization, VP7 selectively induces highly specific antibodes.

In some cases, a rotavirus strain will not react clearly in reciprocal neutralization assays with hyperimmune antiserum. This is usually because the two viruses being compared possess distinct immunologic forms of VP4 (the second outer capsid protein), which is also a neutralization antigen (9). Because the genes that encode these separate neutralization antigens can segregate (reassort) independently, it is not suprising that some virus isolates can possess heterologous neutralization (VP4, VP7) antigens (9). Classification of rotaviruses by a binary system (similar to that used for influenza viruses), in which distinct serotypes of VP4 and VP7 are named, has been accepted (10, 7, 11). A lack of readily available typing serum of MAbs to different VP4 types has hampered classification of VP4 (P) serotypes (P serotype because VP4 is protease-sensitive). Instead, properties of VP4 have been studied by sequence analyses, and current evidence suggests the existence of at least 19 different genotypes of VP4. Genotypes of VP4 and VP7 are determined by sequence analysis whereas serotypes are determined by reactivity with polyclonal or monoclonal antisera.

2. STRUCTURE OF ROTAVIRUS.

The morphologic appearance of rotavirus particles is distinctive, and three types of particles can be observed by electron microscopy (Fig.1 and 2). The complete particles (approximately 75 nm in diameter) resemble a wheel with sort of spikes and a well-defind smooth outer rim. The complete infectious particles historically have been called double-shelled particles, although new structural data show that they have three-layers. Triple-layered particles are infectious and they are composed of the viral proteins

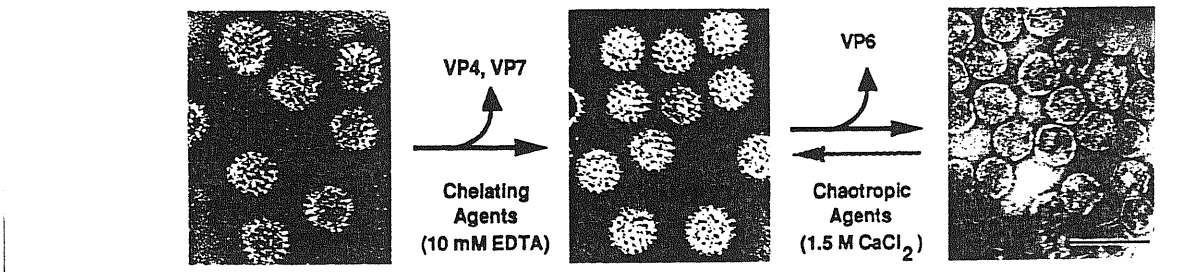
VP1, VP2, VP3, VP4, VP6, VP7. Double-layered particles (previously called single-shelled particles) lacking the outer shell are often described as rough particles because their periphery shows projecting trimeric subunits of the inner capsid. They consist of VP1, VP2, VP3 and VP6. Single-layered core particles are seen less frequently; they usually lack genomic RNA and are aggregated, noninfectious particles. They consist of VP1, VP2 and VP3.

The three-dimensional structure of triple- and double-layered rotavirus particles (produced without protease treatment) was first determined at 40Å resolution and more recently refined at 26Å resolution using electron cryomicroscopy and image processing techniques (13, 14, 15, 16). These studies unequivocally establish a T=13 icosahedral surface lattice for the two outer layers. A distinctive feature of the virus structure is the presence of 132 large channels spanning both shells and linking the outer surface with the inner core. One hundred twenty channels are along the 6-coordinated centers and 12 are along the 5-coordinated centers. Three type of channels can be distinguished based on their position and size (Fig.2). Type I channels run down the icosahedral fivefold axes, type II channels are those on the 6-coordinated positions surrounding the five-fold axes, and type III channels are those on the 6-coordinated position around the icosahedral threefold axes. The function of these channels is not yet known, but it is possible that they are involved in importing the metabolites required for RNA transcription and exporting the nascent RNA transcripts for subsequent viral replication processes.

Sixty spikes approximately 120Å in length with a knob at the distal end extend from the smooth surface of the outer shell (Fig.2). These protein spikes are situated at an edge of the type II channels surrounding the fivefold icosahedral axes. These spikes are composed of dimers of the hemagglutinin (VP4) (17). Higher resolution reconstructions of native particles and of a spikeless particles (formed by removing VP4 by treatment at high pH) have confirmed that VP4 is the spike (15, 16).

The VP4 spike extends inward into the outer virion surface approximately 90Å and interacts with both VP7 and the inner capsid protein VP6 (Fig.2). The existence of VP4-VP7 and VP4-VP6 interactions imply that VP4 may participate in maintaining the precise geometric register between the inner and the outer capsid as well as affect functional domains. The VP7 outer layer appears to consist of trimers of this protein corresponding to a total of 780 molecules (monomers per particle). VP7 interacts with the tips of the VP6 trimers.

The existence of each shell and the ability of VP7 and VP4 to interact directly with VP6 has been demonstrated by the production of viruslike particles composed of VP2, VP2/6, VP2/4/6, VP2/6/7, and VP2,4,6,7 by expression of the respective proteins (18, 19) or coassembly of purified proteins of each layer onto preformed VP2 particles (20, 21, 22). These results show that the structural proteins have the intrinsic properties of self assembly and suggest high affinity interactions among these proteins.



PARTICLE:	Double-shelled	Single-shelled	Core
STRUCTURE:	Triple-layered	Double-layered	Single-layered
PROTEIN:	VP 1, 2, 3, 4, 6, 7	VP 1, 2, 3, 6	VP 1, 2, 3
BIOLOGIC PROPERTIES:	Infectious, Inactive polymerase	Noninfectious, Active polymerase	Noninfectious, Inactive polymerase

Fig. 1. Structural and biological properties of rotavirus particles. Electron micrographs show typical triple-layered, double-layered, and single layered core particles seen after staining with 1% ammonium molybdate. The double-layered and core particles can be produced by sequential degradation of infectious triple-layered particles as shown. The proteins and biological properties of the particles are detailed in the text. Bar=100 nm.

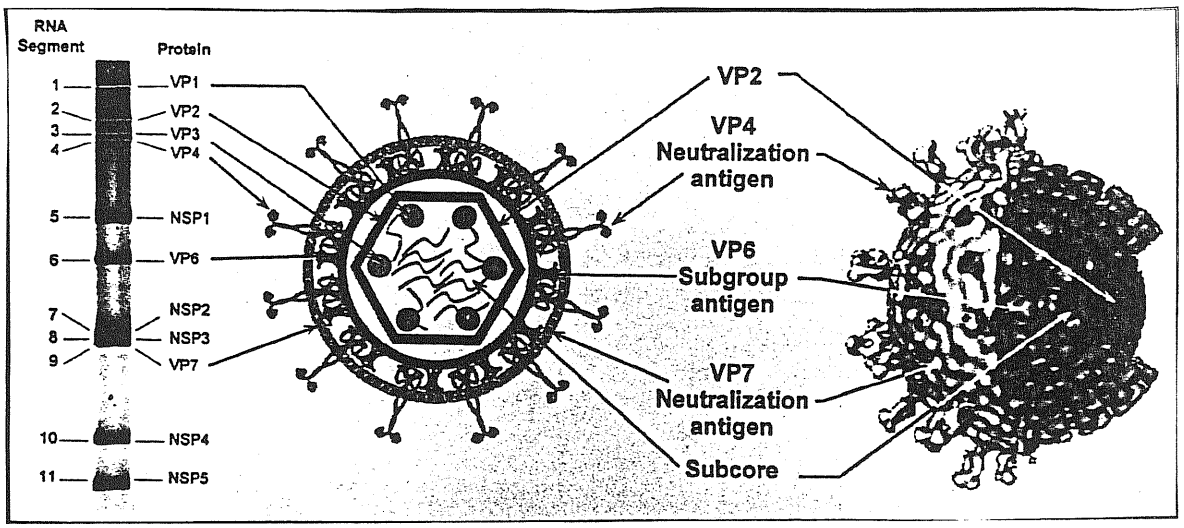


Fig 2. Gene coding assignments and the three-dimentional structures of rotavirus particles. (Left) A polyacrylamide gel shows the 11 segments of dsRNA that comprise the rotavirus genome (SA 11 strain) and the proteins encoded by each of these genes. (Center) A schematic representation of the complete rotavirus particle. The proteins in the different shells are indicated. (Right) The 28Å three-dimensional structure of the complete rotavirus particle, in which a portion of the outer shell mass and inner shell mass have been removed, showing the middle and inner shell. This structure was determined by image processing of electron micrographs of particles embedded in vitreous ice .

3. GENOME STRUCTURE.

The genome of rotaviruses consists of 11 discreate segments of dsRNA. The genomic RNA can be easily extracted from semi-purified rotavirus particles, separated by polyacrylamide gel electrophoresis (PAGE) (Fig.3) and visualized by ethidium bromide or silver staining (117). The RNA segments are numbered 1-11 according to their order of migration, RNA1 being the slowest migrating RNA segment and RNA11the fastest.

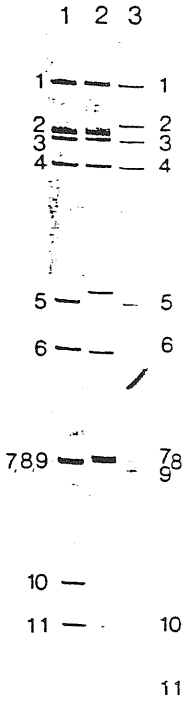


Fig.3 RNA profiles of human group A rotaviruses of subgroup I serotype 2 (*lane 1*) and subgroup II serotype 1 (*lanes 2 and 3).* RNA segments (1-11) are indicated. 3% PAGE; silver staining.

The nucleotide sequence of all of the 11 rotavirus RNA segments for several rotavirus strains is now known. The sequences from different rotavirus strains have shown general features (Fig.4) about the structure of each of the genome segments. Each

RNA segment starts with a 5' guanylate followed by a set of conserved sequences (10nt long) that are part of the 5' noncoding sequences. An open reading frame (ORF) coding for the protein product and ending with the stop codon follows, and then another set of noncoding sequences that contain a subset of conserved terminal 3' sequences (8nt long) and end with a 3'terminal cytidine is found. The lengths of the 3' and 5' noncoding sequences vary for different genes and no polyadenylation signal is found at the 3' end of the genes. All of the sequenced genes possess at least one ORF after the first initiation codon.

The dsRNA segments are base paired end to end and the plus-sense strand contains 5'cap sequence $m^7GpppG^{(m)}GPy$ (28, 29). Similar features of the RNA termini (capped structures and 5' and 3' conserved sequences) are found in the primary structure of the genome segments of other viruses (e.g., reovirus, cytoplasmic polyhedrosis virus, orbivirus) in the family Reoviridae (30, 31, 32) and in other virus families with segmented genomes (Orthomyxovyridae, Arenaviridae, and Bunyaviridae). The strong conservation of terminal sequences in genome segments suggests that they contain signals important for transcription, RNA transport, replication, or assembly of the viral genome segments.

The eletrophoretic migration rate of cognate (segments encoding the same protein) RNA segments in different virus strains often shows heterogenity. In contrast, sequence data show that cognate genes from different strains usually contain the same number of nucleotides. Therefore, the heterogeneity in RNA segment mobility, observed among the cognate RNA segments of different virus strains, is atributable to sequence differences and secondary or tertiary structure that remain during electrophoresis of these segments.

One of the mechanisms which generates heterogeneity in RNA segment mobility in cognate RNA segments in different virus strains is genome rearrangements

(concatamerization/deletion). Genome rearrangements are thought to be a third mechanism of evolution (in addition to antigenic shift and drift) of rotavirus (46).

In most cases, the rearrangements have resulted from a head-to-tail duplication that occurs immediately downstream from the stop codon of the normal ORF; hence, the rearranged segment retains its capacity to express the normal protein product. This indicates that the rearrangement of the sequences in a segment has left the normal reading frame intact and its expression unaltered. In most cases, the profiles of virus-coded proteins in cells infected with rotaviruses with rearranged genomes are similar to those seen in cells infected with standard rotavirus strains (38, 39, 34, 42, 43, 44, 45).

In viruses with genome rearrangement, typical RNA segments are missing and these are replaced by additional more slowly (or reary more rapidly) migrating bands of dsRNA. The slowly migrating bands represent concatameric forms of dsRNA containing sequences specific for the missing RNA segments (33, 34, 35, 36, 37). The more rapidly migrating bands appear to represent deletions. Isolates with rearrangements in segments 5, 6, 8, 10, and 11 have been characterized, with the greatest number having rearrangements in segment 11. It is unknown if the rearrangements in segment 11 occur more frequently or if viruses with a rearranged segment 11 have some selective advantage (better grouth or stability), so they are more easily detected .

Viruses containing rearranged genome segments are generally not defective, and the rearranged segments can reassort and replace normal RNA segments structurally and functionally (38, 39, 40). Biophysical characterization of such particles has shown that up to 1800 additional base pairs can be packaged in particles without causing detectable changes in particle diameter or apparent sedimentation values. However, the density of particles containing rearranged genomes may be increased, and the increase in density must be directly proportional to the number of additionally packaged base pairs (41). These results indicate that rotaviruses have considerable capacity to package aditional

genomic RNA, although the upper limit is unknown. Whereas a total of 11 RNA segments are invariantly packaged, there seems to be much less constraint on the length of individual RNA segments assembled into the mature virus particle.

Viral dsRNA forms a dodecahedral structure in which the RNA double helices, interacting closely with the inner capsid layer, are packed around the enzyme complex, which is composed of viral proteins VP1 and VP3, located at the icosahedral 5-fold axes. The ordered RNA accounts for about 4500 out of a total 18525 base pairs in the genome, the largest amount of icosahedrally ordered RNA observed in any virus structure to date. The observed organization of the dsRNA conduces to an orchestrated movement of the RNA relative to the enzyme complex during transcription (26).

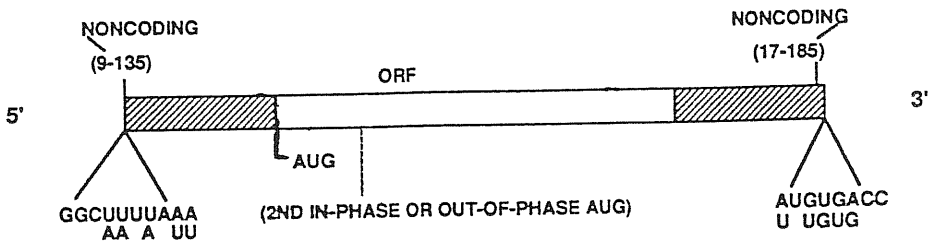


Fig.4. Major features of rotavirus gene structure. The scheme shows the overall structure of rotavirus genes from the published sequences of genes 1 to 11. These genes lack a polyadenylation signal, are A+T rich, and contain conserved consensus sequences at their 5' and 3' ends. The shaded regions represent 5' and 3' noncoding sequences which are part of any rotavirus gene (RNA segment), but can vary in lenght for different genes.

4. ROTAVIRUS PROTEINS-STRUCTURE AND FUNCTION.

4.1 VP1.

VP1 is coded by RNA segment 1 and represent only 2% of all virion proteins (47). It is a subcore protein present in few copies and it has a calculated molecular weight (MW) of 125kD in group A and group C viruses and an apparent molecular MW of 136kD for the group B viruses (88). VP1 there is some similarity with sequences of RNA-dependent RNA polymerases of other dsRNA viruses and picornaviruses (48). The predicted amino acid sequence reveals that VP1 is basic. Several lines of evidence support the hypotesis that VP1 is a component of the viral transcriptase and replicase:

a. VP1 contains sequence motifs shared by RNA-dependent RNA polymerases of other viruses (89, 90, 91).

b. VP1 binds nucleotides and cross-linking of the nucleotide analog azido-ATP to VP1 inhibits viral RNA polymerase activity (92).

c. Baculovirus-expressed core-like particles (CLPs) consisting of VP1 and VP2 can catalyse the synthesis of dsRNA from template mRNA (replicase activity), but CLPs consisting of only VP2 lack such polymerase activity (93).

d. VP1 can bind to 3' end of viral mRNA in the absence of any other viral proteins (94), but the interaction is not sufficient to initiate minus-strand synthesis (23). VP1 purified from open cores by glicerol gradient centrifugation and reconstituted with the VP2-rich portions of the gradient, stimulated levels of replicase activity severalfold. These data indicate that VP1 can bind to viral mRNA in the absence of any other viral proteins and suggest that VP2 must interact with the RNA-protein complex before VP1 gains replicase activity.

4.2 VP2.

VP2 is encoded by RNA segment 2. The gene codes for an ORF of 880 amino acids (aa) (49). VP2 is the major protein component of the core particles and estimates are that particles contain approximately 200 molecules of VP2. Each of the various VP2 proteins is proline-rich (>3.6% proline) and most of these proline residues are conserved. A repeat (aa 55-89) of positively and negatively charged residues in a region predicted as helical is conserved among the VP2 of the group A viruses, but not in the group C virus VP2. The NH_2-terminal half of the protein exhibits two stretches of sequences containing leucines in every seventh position ("leucine zipper") (49).

VP2 is the only protein in the central core with demonstrated nucleic acid binding activity, although this was not sequence-specific (95). It has been shown that VP2 is a component of the core replicative intermediates (RI of 310S), but VP2 is absent from the smallest pre-core RI (220S) (96, 97).

Expression of VP2 in the baculovirus system has shown that this protein, in the absence of the other viral gene products, self-assembles into particles that have a morphology identical to cores (98). These particles are devoid of nucleic acid. In this system, two degradation products of VP2 are seen and these are similar in size to the degradation products identified in empty particles purified from rotavirus-infected cells (98, 99, 100). These results suggest that in the absence of RNA, VP2 presents some region that is specifically suseptable to protease, or that VP2 itself has autoproteolytic activity under certain circumstances.

4.3 VP3.

VP3 is encoded for by RNA segment 3. It is a minor subcore protein that is the most difficult to identify clearly. SA11 genome segment 3 codes for a protein with an apparent molecular weight of 88kD (101). VP3 of porcine Cowden strain is encoded by the gene segment 4 (102) and is shorter than its group A counterparts (692 aa MW 81kD for group C-VP3 versus 835 aa, MW 98kD for group A-VP3).

In vitro , rotaviruses transcribe capped mRNA containing a ^{7}mGpppGmp cap at the 5' end (103). Using purified viral particles, only the core polypeptide VP3 exibits the ability to form a covalent complex with [a-^{32}P] GTP like other guanylyltransferases. VP3 expressed alone in the baculovirus system covalently binds GTP in the absence of other viral proteins, which directly shows that VP3 is the rotavirus guanylyltransferase (104).

4.4 VP4.

VP4 is coded for by RNA segment 4.VP4 genes of various rotavirus strains have been sequenced and sequence comparisons have allowed the general features of the protein product to be deduced.

There is a long ORF coding for 776 aa in animal rotaviruses and for 775 aa in human rotaviruses. Recently, an animal rotavirus VP4 with 772 aa was described (106). Proteolytic cleavage occurs at arginine residues 241 and (preferentially) 247 (84) which are conserved in all rotavirus VP4 sequences obtained so far (85). This cleavage generates two polypeptides VP5* and VP8* which remain associated after the cleavage. Some human strains may have additional cleavage sites after aa-position 246 which has been suggested to be correlated with virulence (64).

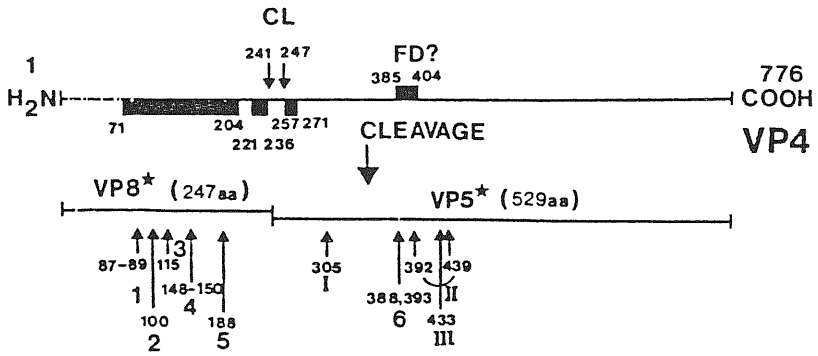


Fig 5. Characteristics of VP4 on the basis of sequence, protein and biological data. The protein precursor is 776 aa long. The two proteolytic cleavage sites (CL) and the putative fusion domain (FD) are indicated *above the line*. The boxes *below the line* show the positions of a highly variable region (aa 71-204) and of two conerved regions around the cleavage sites. The cleavage products VP5*and VP8* are delineated as are the positions of neutralization epitopes 1-6 (367) and I-III (368).

There are conserved cysteine residues in position 216, 318, 380, and 774 of all rotavirus strains (64, 65). Most animal strains contain an additional cystein residue at position 203 which might be important for stabilization of protein structure via disulfide bondage (65). Vatious positions contain conserved prolines (amino acids 71, 76, 77, 110, 225, 226, 235, 334, 390, 395, 434, 451, 455, 482, 524, 669, 716, 749, 761).

VP4 is a non-glycosylated protein of the outer capsid. VP4 has been implicated in several important functions such as cell penetration, hemagglutination, neutralization and virulence. Within serogroup A, and very probably in the other serogroups, VP4 induces neutralizing antibodies that may allow serotypes to be defined (58, 105). It has been proposed that rotaviruses characterized by differences in the neutralization based on VP4 be referred to as P (for protease-susceptible) serotypes. VP4 makes up the 60 spikes that extend from the outer capsid surface (54, 127). Treatment of purified particles at pH 11.2 will release VP4 (or its two cleavage products VP5* and VP8*) and yield smooth double-shelled particles. Such smooth spikeless particle can be decorated by a MAb specific for VP7, implying that the conformation of epitope(s) on the VP7 that forms the smooth surface of the particle does not change significantly by removal of the VP4 spikes (197).

The predicted secondary structure of group A, B and C rotavirus VP4 confirms that for all these viruses the amino terminal 60% of the protein is rich in beta strands that alternate with turns and short stretches of alpha-helixes. The remaining carboxy terminal part of VP4 is predicted to be rich in alpha-helixes. Particulary, a possible heptad repeat pattern has been identified in the region between aa 494 to 554 (102, 108). This region is well conserved through the three virus groups and predicted as an alpha-helix (198) moreover, a shorter heptad repeat can be identified for the three virus groups. This heptad repeat is a good candidate for the VP4 oligomerization domain.

Recently the gene 4 of the bovine strain B223 has been sequenced and found to have unique features (106). This VP4 is shorter (772aa) and it shows only 70-74% homology with other VP4s so far sequenced. This protein has two continous aa deletions within the trypsin cleavage region but the trypsin cleavage site appears to be intact. Apparently other sites in VP4 are important in maintaining the proper conformation of the cleavage site. Comparison of the conserved region flanking the trypsin cleavage site shows that the region 5' to the cleavage has a very low similarity 66% to other rotavirus strains, while the flanking region 3' to the cleavage site shows comparable conservation with that of others rotavirus strains. The lack of the conserved proline in the region 5' to the cleavage site suggests a possible altered local conformation of this site in B223 VP4 (106). A more unexpected result is that viruses containing this unusual B223-like VP4 also have been isolated from children in India (119). These results are the strongest indication that animal viruses can infect humans.

Information on the antigenic and biologic properties of VP4 has begun to be uncovered by expression of this protein in different systems. The complete VP4 from three group A viruses (SA11, RRV, OSU) has been expressed in high yield in insect Sf9 cells (109-111). The baculovirus-expressed VP4 protein binds to erythrocytes and has hemagglutinating activity. The VP4 produced in this system also induces antibodies which inhibit hemagglutination of the homologous strains and neutralize viral infectivity. MAbs directed at VP8* efficiently prevent binding of radiolabeled virion to MA104 cells monolayers, to an extend and at a concentration comparable to those required for neutralization of infectivity (112). Conversely, MAbs recognizing VP5*, show little or no inhibitory effect on virus binding to cells. MAbs directed at VP8*, but not those recognizing VP5*, mediate release of radiolabeled virus from the surface of the cells (112). This last result suggests that VP8* could be the part of the viral protein that interact

with the cell receptor, with VP5* being implicated in the interaction with the lipid bilayer (112).

A putative fusion region (aa 384-404 for SA11) was identified in group A VP4 by sequence comparison with the E1 glycoprotein of Sindbis and Semliki Forest virus (113). The short stretch FQIPVGA in the middle of this region is highly conserved between the VP4 of group A and C viruses, and it is tempting to propose that these amino acids are the residues used for the putative fusion activity by group A and C viruses.

Direct evidence of fusogenic activity of this region of VP4 is still missing, but several resalts support the role of VP4 in virus-cell membrane interactions. First, the neutralizing MAb (2G4) that was localized to the ends of the spikes by electron cryo-microscopy (17) binds to this putative fusion region. Second, purified trypsinized viral particles were shown to interact with lipid bilayers using liposomes as a model system (114). Rotavirus particles with uncleaved VP4 do not interact with liposomes, but treatment *in situ* of these particles with trypsin restores the interaction with the liposomes. The interaction is maximal at neutral pH. These data support the view that rotavirus enters the cell through direct penetration of the plasma membrane and that cleavage of VP4 is necessary for the interaction of the virus with the membrane (115).

Baculovirus-expressed VP4, like VP4 of infectious double-shelled particles, bind to 300- and 330-kDa proteins present in the brush border membranes of murine enterocytes (118). The ability of rotavirus to bind via VP4 to large brush border membrane glycoproteins correlates with *in vivo* rotavirus cell tropism and host range restriction (119).

The sequencing of escape mutants selected with neutralizing MAbs has identified amino acids involved in homologous and heterologous neutralization. VP8* contains predominantly type-specific sites, while cross-reactive sites have been located on VP5* (58, 120). No amino acids changes were found within or close to the cleavage sites

which have been suggested to represent antignic sites (121). A neutralizing MAb directed to VP4 of rotavirus strain K8, which has unique VP4 neutralization epitopes, has been idntified. Escape mutants to this MAb contain a mutation at position 394 (His changed to Tyr) which is included in the major cross-reactive neutralization region identified in other viruses (122). Of interest, the group C Cowden strain also has an His residue at aa 394, as does K8. Taken together, these data highlight aa 394 as an important region in virus neutralization and possibly in entry into cells.

The interaction of VP4 with other structural or nonstructural proteins has been studied by various strategies. In infected cells, 16S to 20S complex which contained the rotavirus outer shell proteins VP7 and VP4 cross-linked to NSP4 was observed (123). In the absence of Ca^{++} or presence of tunicamycin, VP7 was excluded from these hetero-oligomers. In the presence of Mn^{++}, VP4 did not form a hetero-oligomeric complex with NSP4 and VP7 (124). Complexes of VP4, NSP4, and VP7 have been detected in the endoplasmic reticulum membrane; such complexes apparently participate in the budding of the single-shelled particles into the lumen of the endoplasmic reticulum where maturation to double-shelled particles occurs.

4.5 VP6.

VP6 is coded for by RNA segment 6 and is the most abundant protein providing 50% of the weight of virions (47, 54).

High resolution cryo-microscopy studies have revealed that the inner capsid of rotavirus has a "bristled" outer surface composed of 260 trimeric-shaped columns of density, atributed to VP6 (54, 127). Thus, there are 780 molecules of VP6 per virion. The trimers of rotavirus VP6 are stabilized by non-covalent bonds that resist threatment with 1% SDS at room temperature but not at 100^0C. The existance of intrachain disulfide bonds

has been suggested because changes of the electrophoretic migration of monomers and trimers are seen in polyacrylamide gels depending on whether the gels contain a reducing agent such as beta-mercaptoethanol (128).

VP6 from different strains display a diferent apparent MW, but they have very similar calculated MWs based on sequence informtion. Since these proteins have very similar primary structures, these biochemical differences must be due to a differential resistence to the denaturing effects of SDS or to differential post-translational modifications. It is noteworthy that VP6 is myristoylated (129), and differences in molecular weight also can reflect differences in myristoylation.

The sequence of VP6 has been determined for 9 strains of group A, two isolates of group B (130, 131) and two isolates of group C rotaviruses (132, 133). VP6 is encoded by segment 6 for the group A, B and C viruses, and by segment 5 for the group B and C viruses. As noted above for the virious other genes that have been sequnced for the group A, B and C rotaviruses, there is more similarity between group A VP6 and group C VP6 (59% similarity, 42% identity) than between the VP6s of group A and B viruses (43% similarity, 16% identity).

Sequence analysis reveals that the VP6 proteins of the viruses in the 3 groups are particularly hydrophobic, and each contains a single hydrophpbic region (aa 99-110). Each VP6 contains a high percentage of predicted beta structure and a very low percentage of alpha helix. VP6 is proline-rich (5.2%) and it contains 3 (group A and C) or 6 (group B) cysteins. A multiple alignment of all the published VP6 sequences (119) indicates that a single cysteine and 11 prolines are conserved between group A and B virus strains. However, for the 3 virus groups, there is a clear accumulation of prolines near the carboxyl end.

VP6 is the primery antigen detected in most diagnostic assays and several epitopes have been identified on VP6. These epitopes are not involved in virus

neutralization. VP6 contains common (cross-reactive) epitopes called group antigens that are shared among all viruses of a given group. The presence of common epitopes on VP6 is not a unique property of this protein. However, because of the abundance of VP6, detection of such epitopes has been very easy. Therefore, reference to VP6 carrying "the common antigen" really is not correct. One immunodominant site of VP6 that contains group specific epitopes has been mapped between the amino acid residues 48 to 75 (135).

Other epitopes, called subgroup antigen, have been defined serologically on VP6 and have been used as an epidemiological tool because they can identify different virus strains (134). Each subgroup has evolved separately.

VP6 also interacts with VP2. Sf9 cells co-infected with baculovirus recombinants containing gene 2 and gene 6 express VP2 and VP6 that spontaneously assemble into particles of VP2 and VP6 (98). These VP2/6 particles are morphologically identical to single-shelled particles that lack genomic RNA (empty particles) (98). Coexpression of VP2 from group A rotavirus (Bovine RF strain) and VP6 from group C (Porcine Cowden strain) in the baculovirus results in the formation of chimeric empty single-shelled particles (200) The assembly of these chimeric particles demonstrates conservation of group A and group C VP6 structures required for interactions with VP2. In terms of the primary structures, one or two of the conserved regions in VP6 (aa 1-140 and aa 353-394) could be implicated in these interactions. The region between group A VP6 aa 251 and 397 is reported to be essential for binding to cores to form single-shelled particles (136).

To localize the domain(s) of VP6 required for assembly and trimerization, amino-and carboxyl-trancated species of VP6 were examined by electrophoretic assay for the ability to trimerize *in vitro* . The results showed that the domain for trimerization residues near the center of VP6 (between aa residues 105 and 328) while the domain for assembly is located between aa 251-397. The fact that some truncated species of VP6 were

able to bind to core particles, but were unable to form trimers *in vitro* , suggests that trimerization of VP6 is not a prerequisite for the assembly of single-shelld particles (136). VP6 can self-assemble *in vitro* and form spherical-like particles (137). These particles may possess an inherent capacity to target to cells of the immune system (138) and have been used as a carrier of peptides derived from VP7 and VP4 (201).

It has been demonstrated that removal of VP6 results in the loss of the transcribtase activity of single-shelled particles. However, by itself, VP6 has no polymerase activity. A major question is whether VP6 has a role in the transcription process via the interaction with the proteins involved in mRNA synthesis, or via a direct interaction with the genomic dsRNA template or with the nacent transcripts. Apparently, VP6 does not bind nucleic acids either in the viral particle, in infected cells, or in the active transcription complex (139, 140). Previous results on the morphogenesis of particles have demonstrated that VP6 is added to particles after completion of replication of the genome, suggesting that VP6 is not part of the replicase (119).

The interaction of VP6 with its specific receptor (NSP4) on the ER also has been characterized (141, 142). The interaction between VP6 present on the single-shelled particles and the receptor is enhanced by the presence of Ca^{++} and Mg^{++}. A monoclonal antobody specific for VP6, and VP6 derived from virus, blocks the ability of membranes bearing NS28 to bind to a single-shelled particles (141, 142). Purified enveloped intermediate particles are essentially composed of VP1, VP2, VP4, VP7 and NSP4 (183). These studies suggest the addition of the outer capsid proteins (VP7 and VP4) to single-shelled particles is directed by a VP6-NSP4 interaction which occurs during the budding process of single-shelled particles through the ER membranes.

Studies in a SCID mouse model suggest that VP6 possesses a cross-reactive cytotoxic T lymphocyte (CTL) epitope. Transfer of $CD8^{+}$T lymphocytes from mice immunized with recombinant VP6 can significantly reduce the level of replication of

rotavirus in infected SCID mice (143). However, cross-reactive CTLs recognize target cells expressing VP6 to a much lower extend than target cells expressing VP7 or VP4 (144). These results, and reported partial protection provided by baculovirus derived VP6 (145), suggest that VP6 may play a role in protection from infection.

4.6 VP7.

The genes coding for VP7 are either RNA segment 7 (resus rotavirus (RRV), serotype 3), RNA segment 8 (bovine rotavirus, serotype 6) or RNA segment 9 (simian rotavirus SA11, serotype 3).

The nucleotide sequences of VP7 genes of most serotypes have been determined and the following common fetures arise: The nucleotyde sequence reveals two ORFs of 326 and 286 aa-length, respectively. The ORFs are in frame and are both used in SA11-infected cells (59). They start with hydrophobic sequences which could be signal sequences directing the nascent VP7 to the endoplasmic reticulum. Both signal sequences seem to be cleaved at amino acid 51 (Gln) (60). The potential glycosylation site at amino acid 69 is present in almost all VP7 molecules (except for the calf rotavirus strains NCDV and RV). Other potential N-glycosylation sites are in amino acid positions 146, 238 and 318.

VP7 is the most abundant protein of the rotavirus outer shell and forms the smooth external surface of mature virions. VP7 does not form the viral spikes, although it is thought to interact closely with VP4 in an unknown way, based on several observations. First, experiments performed with different combinations of heterologous VP7 and VP4 and with reassortants, have shown differences in the stability of the outer capsid of the assembled viral particles (126, 146, 147). Moreover, MAbs against VP7 inhibit hemagglutination. This apparently is caused by steric hidrance, because hemagglutination

is known by genetic analysis to be a property of VP4. Finally, the binding of some MAbs to escape mutants that are mapped either to VP7 or VP4, may be influenced by the structure or a mutation present in the other protein; these results may reflect changes induced in an interaction domain (203).

Early three-dimensional structural studies (148, 149) and more recent studies using electron cryo-microscopy have confirmed that the outer capsid shell of rotaviruses consists of 780 molecules of VP7 arranged around 132 aqueous holes (54, 127). The outer capsid reveals a closely packaged network of 260 triangular-shaped motifs, which suggests that VP7 may exist as a multimer in the outer capsid (54, 127).

VP7 is a glycoprotein that contains only N-linked high manose oligosaccharide residues which are modified by trimming in the ER (58). VP7 is an integral membrane protein with luminal orientation, which is translated by ribosomes associated with the ER and is subsequently translocated into the lumen of the ER. The native viral VP7 is a highly immunogenic and induces mostly serotype-specific, but also cross-reactive neutralizing antibodies. Fourteen deferent VP7 serotypes have been identified (VP7 or glycoprotein, Gtypes) comprising human and animal species of rotaviruses (58).

The aa-sequence of VP7 differs highly among viruses in the three groups. The group C VP7s are closer to group A VP7 and share only a 30% aa homology, while the human and rat group B VP7s respectively, are 28 and 18% homologous to group A VP7. However, the overall structure is similar, especially between group A VP7 and group C VP7, but also with group B VP7.

The VP7 in the three groups have a different number of cysteins (8 for group A, 11 in group C, and 6 in group B), but these are conserved within each group, and two of them (corresponding to positions 196 and 205 of the SA11 VP7) located in an intergroup conserved region, are conserved between VP7 of the three virus groups.

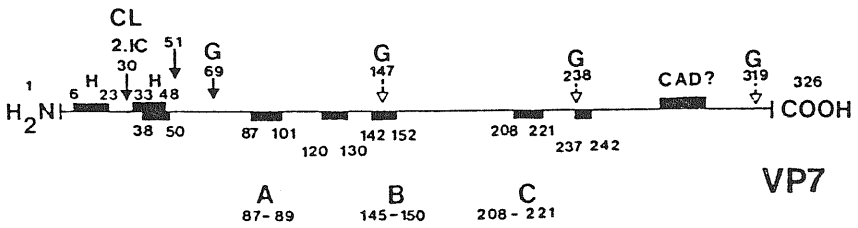


Fig 6. Characteristics of VP7 on the base of sequence, protein, and biological data. The protein is 326 aa long. *Above the line* the two hydrophobic (H) sequences, the second initiation codon (2.1C), the signal peptide cleavage site (51), four potential glycosylation sites (G), of which G69 is normally used, and the suggested cell attachment domain (CAD) are indicted. The *blocks below the line* delineate variable regions, and the three areas of neutralization epitopes (A, B, C) are indicated. Site A binds serotype-specific and cross-reactive MAs, site C only serotype-specific MAbs.

The number and location of the putative glycosylation sites is variable within the group A VP7s and it is probably the same within the other groups. Glycosylation does not appear to be critical for virus infectivity, at least in tissue culture (150).

Three regions of VP7 are especially conserved between the viruses in the 3 groups. The conserved regions are:

a. the presence of an amino-terminal hydrophobic signal;

b. a region between aa192 and 231 of the group A VP7;

c. a carboxy terminal region located at aa 278-309 of group A VP7.

The first intergroup conserved domain includes the amino-terminal hydrophobic sequence. The group A VP7 gene possesses two potential in-frame initiation codons, each preceding a hydrophobic domain, called H1 and H2. Mature VP7 is derived from a precursor that is cleaved by signal peptidase at a site located between Ala 50 and Gln 51 (151). This cleavage removes both amino-terminal hydrophobic sequences and creates the new amino-terminus of the virion-assossiated VP7. The processed products translated from either the first or second AUG are the same, which means that the first initiation codon is not needed. It remains unknown why the two hydrophobic sequences in the amino terminus are conserved in all Group A VP7.

Two in-phase initiation codons also are present in the group C strains preceding each of the two hydrophobic sequences in the amino terminus of group C VP7 (152). Only one AUG is found in group B VP7s preceding the single hydrophobic sequence (153). A signal cleavage site is predicted in group C VP7 (Gly 49 and Gln 50) and in group B VP7 (Ala 15 and Gln 16).

Although it is not known how VP7 is anchored, it is classified as a membrane-associated protein since it is retained in the ER. An initial question was what mechanism accounts for the retention of this glycoprotein in the ER. VP7 does not contain the known KDEL ER-retention sequence (58). It has been found that the sequences necessary for

group A VP7 retention lie within the first 111 residues of VP7 with two regions, aa 51-61 and 62-111, being important for retention (154). Other data suggest that an interaction between the cleaved H2 signal peptide and sequences within the first 111 residues may be responsible, directly or indirectly, for ER retention (155). A search in the Prosite Database shows that group A VP7 bears a consensus sequence LPxTG(STGAE) for an anchoring hexapeptide that has been proposed as being responsible for a posttranslational modification necessary for proper anchoring of the protein which bear it to the plasma membrane. This highly conserved sequence LPITGS found between aa 57 and 63 of group A VP7, is located in the region reported to be critical for ER retention of this glycoprotein. However, a similar sequence is not present in the amino-terminus of VP7 of the group C and group B viruses.

The intergroup conserved sequence located at aa 192 to 231 of group A, aa 197-236 of group C (51.3% homologous) and aa 131-170 of group B (53.8% homologous) is of particular interest because this correspond to one of the region known to be important in neutralization of group A viruses. Two Cys (aa position 191 and 196 of group A) in this region are conerved in the 3 groups. Three mejor antigenic regions, called A, B and C, have been mapped to group A VP7 (203). The intergroup conserved region in group A includes the antigenic region C (aa 208-221), described as one of the most immunogenic sites in group A VP7, where several escape mutants have been mapped. This C region is thought to be involved in determining a conformational epitope together with the A antigenic region (aa 86-101). More recent studies have indicated that the C and A region also interact with the B antigenic region (aa 142-152) (156, 157). The conformation of this complex domain is critical for viral neutralization, given that the neutralization escape mutants of several rotavirus strains map in region A (especially aa 94) and C, or both. In the intergroup conserved region, the 2 group C proteins are almpost 100% homologous, and both group B proteins share 78.8% homology which is much

higher than the homology shown in the rest of the group B VP7 protein. The importance of this region for group A viruses has been emphasized by reports that one-way cross-neutralization results may reflect differences in the sequences in VP7 epitope A, rather than the presence of a distinct VP4 on viruses (158).

The third similar domain between VP7 of the 3 rotavirus groups is located in the carboxy-terminal portion of the protein that includes a stretch of aa that have been proposed to be involved in binding to cells. VP7 has been proposed as the cell attachment protein by several authors (159, 160). A region at the carboxyl terminus of VP7 has been hypothesized to be an attachment region, because a peptide containing aa 275-295 and a monoclonal antibody to this peptide, blocked adsorbtion of virus to cells (160, 161). The result with VP7 can be interpreted as a steric effect in view of new data available on VP4 that assign the cell attachment function to VP4 (119). However, a carboxy terminal region that includes some of these aa, found between aa 283-315 of the group A VP7 sequence, is one of the two regions found conserved in the three groups of rotavirus. The function of this conserved domain is unknown, but it is interesting to ask if it is exposed on the surface of particles and if it may interact with VP4.

A putative Ca^{++} binding site predicted for group A VP7, at aa 124-125, is not predicted in group B VP7 or group C VP7. Calcium is thought to be critical for virion stability in group A. Differences in the calcium-binding capacity of distict strains may exist, but the outer capsid of viruses in all three rotavirus groups is removed by treatment with EDTA (157). Non-group A rotaviruses also have been found to be more unstable upon storage, with most of the viral particles being single-shelled. So far, the reason for this difference in stability is unknown. However, uncoating and assembly if the outer capsid could be directed by the various free Ca^{++} concentrations in the cytoplasm and in the ER. Whether this is mediated only by VP7 or by other viral proteins remains to be determined.

VP7 is thought to be incorporated into virions somehow during the maturation and budding of single-shelled particles in the ER. Two pools of VP7 have been detected in the ER, and they can be separated biochemically or by using antibodies that can distinguish between the membrane associated and the particle-associated form of VP7 (163). Experiments based on the use of membrane-permeable and impermeable crosslinking reagents have suggested that during the virus maturation process, VP7 shifts its position from its luminal association with the ER membrane, to the interior of the intermediate enveloped particles (163).

4.7 NSP1.

Only three group A genes coding for the nonstructural protein NSP1 (formely called NS53), and one group C gene have been sequenced to date (164-166). Comparative sequence analyses have shown that this gene apparently is the least conserved of all the group A rotavirus genes (166-168). SA11 NSP1 has a calculated MW of 58.4kD and is coded by segment 5. For porcine Cowden strain, the calculated MW is 46.4kD and is coded by segment 7. Sequence analysis reveals a repeat of cysteins and histidines that fit well with the consensus for zinc (Zn) finger motif. This motif, [Cys-X_2-Cys-X_5-Cys-X_2-Cys] where X indicates any other amino acid, is conserved in the three published sequences. In the bovine RF strain, a similar motif also is present between amino acids 315 and 328. In this motif, key residues (cysteine or histidines) are conserved, along with several other amino acids: Ala-Leu, His and Val in the large loop and Trp in the small loop at the carboxy end of the motif.

NSP1 is expressed at very low levels in infected cells, and is detected predominantly early after infection (170). It has been detected in precore complexes which appear at an early stage of virus particle assambly (97). This suggests that NSP1 may play

a regulatory role in the viral replication cycle. Recently it has been shown that the recombinant NSP1 expressed in the baculovirus system binds zink and ssRNA (171).

The fact that the NSP1 sequence is more divergent than any other rotavirus structural or nonstructural gene examined so far is difficult to interpret. This divergency, in conjunction with the observation that viruses carrying a rearranged gene 5 that do not express NSP1 are viable (172) suggest that this gene is dispensable. It also could be hypothesized that the function of NSP1 essentially resides in the conserved Zn binding motif and that function, *in vivo*, could be related to interactions with cellular gene(s).

4.8 NSP2.

This protein is encoded by RNA segment 7 (UK bovine rotavirus) (73), 8 (SA11) (72), or 9 (RRV) (71). NSP2 is a very conserved protein in group A viruses, and most important, it has a high percentage of similarity with the group C protein: 37% identity and 59% similarity. These percentages are significantly than those observed for other proteins whose sequences are available and that have been compared between the two groups. The C-terminus of both proteins is less conserved. The length only is slightly different: 317 for the group A and 312 for the group C protein. The group A NSP2 has 5 conserved cysteins, but only one of them (mapped at aa 6) is conserved in the group C proteins. NSP2 is very basic in both groups and the overall structure is very conserved, including the helix-turn-helix motif predicted in the group A protein between aa 160 and 218 (58).

A Genbank search showed a region in this protein (aa 54-87) similar to bacterial proteases and metalloproteinases (30% identity, 70% similarity). This region is almost 100% conserved in group A and 60% similar to the coresponding region of group

C NSP2 (42.8% identical). It will be of interest to determine if NSP2 has any protease activity.

Very little is know about NSP2. Immunocytochemistry experiments using a monoclonal antibody against a human or bovine group A NSP2 have localized this protein solely to the viroplasms in infected cells (140,173). NSP2 has been found in subviral particles with replicase activity (96, 97, 75) together with NSP3 (NS34), NSP5 (NS26) and NSP1 (NS53).

NSP2 is a nonspecific RNA-binding protein (174). Sedimentation analysis of NSP2 expressed in rabbit reticulocyte lysates by cell free translation and in vTF7-3-infected cells by transfection with a gene 8 containing transcription factor showed that NSP2 assembles into multimers of approximately 10S and that the formation does not require other viral proteins. The 10S multimers were also detected in rotavirus-infected cells, providing evidence that they function in virus replication. By an NSP2-specific RNA capture assay the multimers were shown to possess the RNA-binding activity. DSP-crosslinking of infected cells lysates and immunoprecipitation also revealed that NSP2 interacts with the putative viral RNA polymerase VP1. Analysis of cytoplasmic extracts resolved by sedimentation on glycerol gradient suggested that the VP1-NSP2 complexes are soluble and RNA-free. Complexes formed from NSP2 multimers, VP1, and viral messenger RNA may function to coordinate RNA packaging and the assembly of viral cores (174).

4.9 NSP3.

NSP3 is a nonstructural protein, produced in large amount in rotavirus-infected cells. It is known to be encoded by gene 7 in SA11 strain (140,175), segment 8

(RRV) and by gene 9 in the bovine UK strain. Three cysteins, a positions 123, 139, and 306, are conserved in the simian and the bovine proteins. NSP3 is an acidic protein.

Structural predictions of the sequence of NSP3 (140) suggest this protein consistsof two major domains. The first is a very conseved basic region (aa 83-150). This basic region is predicted to have an alpha-helical structure. A short acidic region follows (9 Glu or Asp over 19 aa) between aa 150 and 169. A consensus sequence (I/L)XXM(I/L)(S/T)XXG, present in NSP3 between aa 104 and 112 is the basic domain, also is present in the ssRNA binding proteins of orbivirus (NS2) and reovirus (sigma NS) (191).

The second domain of NSP3, found in the carboxyl-half, is predicted to contain extended regions of heptadpeptide repeats of hydrophobic residues typical of alpha-helical coiled-coil structures, especially between aa 181-236 and aa 275-305. This last heptad repeat conforms to a leucine zipper of 5 repeats. Sequence homology in this C-terminal half of NSP3 to coiled-coil region of myosin and other proteins like neurofilament triplet L or M proteins, predicts that this region of NSP3 could participate in forming coiled-coil oligomers. The amino terminus of NSP3 contains a stretch of mostly non-charged aa between aa 1-30.

The sequence of porcine Cowden gene 6 protein was reported to be equivalent to the group A NSP3 (177).

NSP3 is found as oligomers when analysed after expression in insect cells in SA11-infected MA104 cells, and in cell-free translation reaction. Investigation of the multiple electrophoretically distinct forms of NSP3 has shown they are all composed of homooligomers. Analysis of deletion mutants constructed and tested for oligomerization showed that both the heptad repeat and the leucine zipper motifs present in the carboxyl half of the protein are responsible for ologomerization (140).

NSP3 has been proposed to have some role in replication and/or assembly of RNA into viral capsids. NSP3 consistently has been found in complexes with replication activity (96, 75). A 31K bovine rotavirus protein bound to both ssRNA and dsRNS in a nonspecific manner (139). It has been assumed in the literature that this protein is equivalent to SA11 NSP3, and, this has been proven directly (140). The basic region is a good candidate to contain the RNA binding domain. Products of the porcine group C rotavirus NSP3 gene bind specifically to dsRNA (178).

Staining of SA11-infected MA104 cell in immunofluorescence assays using a monoclonal antibody shows an unusual distribution pattern for NSP3 (140). Specifically, NSP3 is seen in a complex network of filaments distributed through the cytoplasm instead of in the classic discrete staining of viroplasm seen for other rotavirus proteins. The fact that NSP3 primarily is found associated with the cell cytoskeleton also suggests a role of NSP3 in genome replication and the early steps of viral assembly.

4.10 NSP4.

NSP4 is a nonstructural, transmembrane, glycoprotein encoded by gene 10 of group A rotaviruses. No equivalent of this protein has been sequenced yet in the group B or group C rotaviruses. NSP4 has been associated with the unique feature of rotavirus morphogenesis, that is the maturation of particles by budding through the memebranes of the endoplasmic reticulum (ER). Several studies have demonstrated a receptor role for NSP4, to bind single-shelled particles (ssp) and to translocate these subviral particles across the ER membrane by budding (179, 180). Some studies have suggested VP4 is assembled onto these particles prior to translocation (181).

The primary product of gene 10 is 175 aa long (184), with an apparent MW of 20K. This precursor subsequently is glycosylated at the amino-terminus, at asparagines 8

and 18, giving a mature protein with apparent MW of 28K. A noncleaved signal peptide is present in NSP4. Three hydrophobic domains are present at the amino terminus of NSP4, which is postulated to be retained in the ER membrane, spanning it once. The glycosylated amino-terminal region is oriented on the luminal side of the ER, while the carboxy terminus, which is mostly hydrophilic, extends into the cell cytoplasm.

NSP4 is a well conserved protein. Compared to SA11, the human Wa, the bovine UK and the NCDV proteins show an homology of 87.3%, 96.6% and 92.2%, respectively. Based on cross-linking experiments, it was postulated that NSP4 is an oligomer, probably a dimer or a tetramer (182, 181). Oligomerization of NSP4, VP4 and VP7 has been observed, and in these oligomers, each protein was present in nearly stoichometric amounts (182). It can be speculated that interactions between these three proteins trigger the budding of the bound single-shelled particles. Glycosylation of NSP4 is reported to be necessary for membrane dissolution and final maturation of the virions in the ER, but the mechanism for this is unknown (150).

Heterooligomeric complexes of NSP4, VP7 and VP4 detected in the ER membrane have been suggested to participate in the budding of the single-shelled particles into the lumen of the ER (182, 183). In this budding model, the luminal domain of NSP4 is thought to interact with VP7, while the carboxyl cytoplasmic tail interacts with VP4. Studies done with membrane-permeable and -impermeable crosslinkers confirm this localization. However, during the maturation process, VP7 appears to be repositioned from its localization in the ER lumen to the interior of the viral envelope, because it becomes protected from protease digestion. These some experiment suggest that during the formation of the outer capsid, VP4 within the viral particles may become exposed and thus accessible to proteases and membrane-impermeable cross-linkers. NSP4 remains associated with the transient membrane and is lost along with it apparently after completion of outer capsid assembly (182, 183).

Two functional domains have been mapped to the carboxyl terminal region of NSP4: one domain is for binding of the single-shelled immature particles and another domain is for binding VP4. These two domains have been mapped to aa 161-175 and 112-148, respectively, in experiments using deletion mutants and a monoclonal antibody that maps to the cytoplasmic region (180). There is evidence that VP6 is the viral protein on single-shelled particles that interacts as the ligand with NSP4 (179, 180). It was reported that deletion or a conservative substitution of the C-terminal methionine completely abolished receptor activity of NSP4 in transient expressed system(185).

An interesting observation is that NSP4 is not necessary for the assembly of recombinant rotavirus-like particles made in insect cells infected with recombinant baculovirus that express the rotavirus proteins VP2, VP4, VP6, and VP7 apparently contain the intrinsic signals needed for particle formation.

Recently it has been found that NSP4 also caused an increase in intracellular calcium in insect cells. Purified NSP4 or a peptide corresponding to NSP4 residues 114 to 135 induced diarrhea in young (6 to 10 days old) CD1 mice. This disease response was age-dependent, dose-dependent, and specific. Electrophysiologic data from intestinal mucosa showed that the NSP4 114-135 peptide potentiates chloride secretion by a calcium-dependent signaling pathway. Diarrhea is induced when NSP4, acting as a viral enterotoxin, triggers a signal transduction pathway (83).

4.11 NSP5.

NSP5, the longest product of SA11 gene 11, is a nonstructural protein relatively well conserved in group A rotaviruses. Although the predicted MW calculated from the gene sequence is 21.725, the primary product of gene 11 has an apparent MW of 26K, which then is processed to a 28K polypeptide.

Two kinds of modification have been reported for NSP5. It was phosphorylated (186) at its 26K and 28K forms and that it had a second uncharacterized type of modification, which has been subsequently found to be O-glycosylation (187). NSP5 is O-glycosylated through the addition of simple N-acetylglucosamine. The 26K-to-28K shift was attributed to this rare type of O-glycosylation that occurs on ony a few nuclear and cytoplasmic proteins (66). Several lower MW polypeptides present in infected cells and cell-free translation reactions and serologically related to NSP5 also are phosphorylated. One of these polypeptides is a 20K product found to be translated from a second in phase methionine at position 52 (188) that encoded a protein of 148 aa. This protein is modified to a 22K polypeptide. A second protein, called NSP5a, is thranslated *in vivo* and in cell-free system as a 12K polypeptyde (188). NSP5a is translated from a shorter alternative out-of-phase ORF that starts at nucleotides 80-82 and encodes a protein of 92 aa. The function of the NSP5a protein remains unknown.

The genes that encode NSP5 from two group B and two group C rotaviruses have been sequenced (Fig. 7). In the case of the Group B, the strains sequenced were the human ADRV (189) and the rat IDIR strains (190), in which the NSP5 equivalent is encoded by gene 11, as in group A rotaviruses. The group C viruses whose NSP5 has been sequenced are the human Bristol (181) and in porcine Cowden (191) strains. For these two strains, the NSP5 protein is encoded by gene 10. A second ORF that could encode NSP5a in the gene coding for NSP5 has not been reported for the non-group A rotaviruses.

Comparisons of the amino acid sequences of these proteins of viruses in different groups yield interesting results. The length of the proteins coming from the different groups is strikingly different. Group A NSP5 is 198 aa long, group B ADRV has 170 aa and group B IDIR has 175 aa, group C Bristol strain and group C Cowden strain are 212 and 210 aa long, respectively. This results in a low overall nucleotide and amino

acid homology. Despite this, ther are some features shared by NSP5 from viruses in all the three groups. All the proteins have a high percentage of serines and threonines (23.7% for SA11, 16.3% for Bristol strain, 20.5% for Cowden strain, 15.9% for ADRV strain and 18.4% for IDIR strain). The NSP5 proteins are mostly hydrophilic. The group A and B NSP5s are neutral, but the group C NSP5 is acidic.

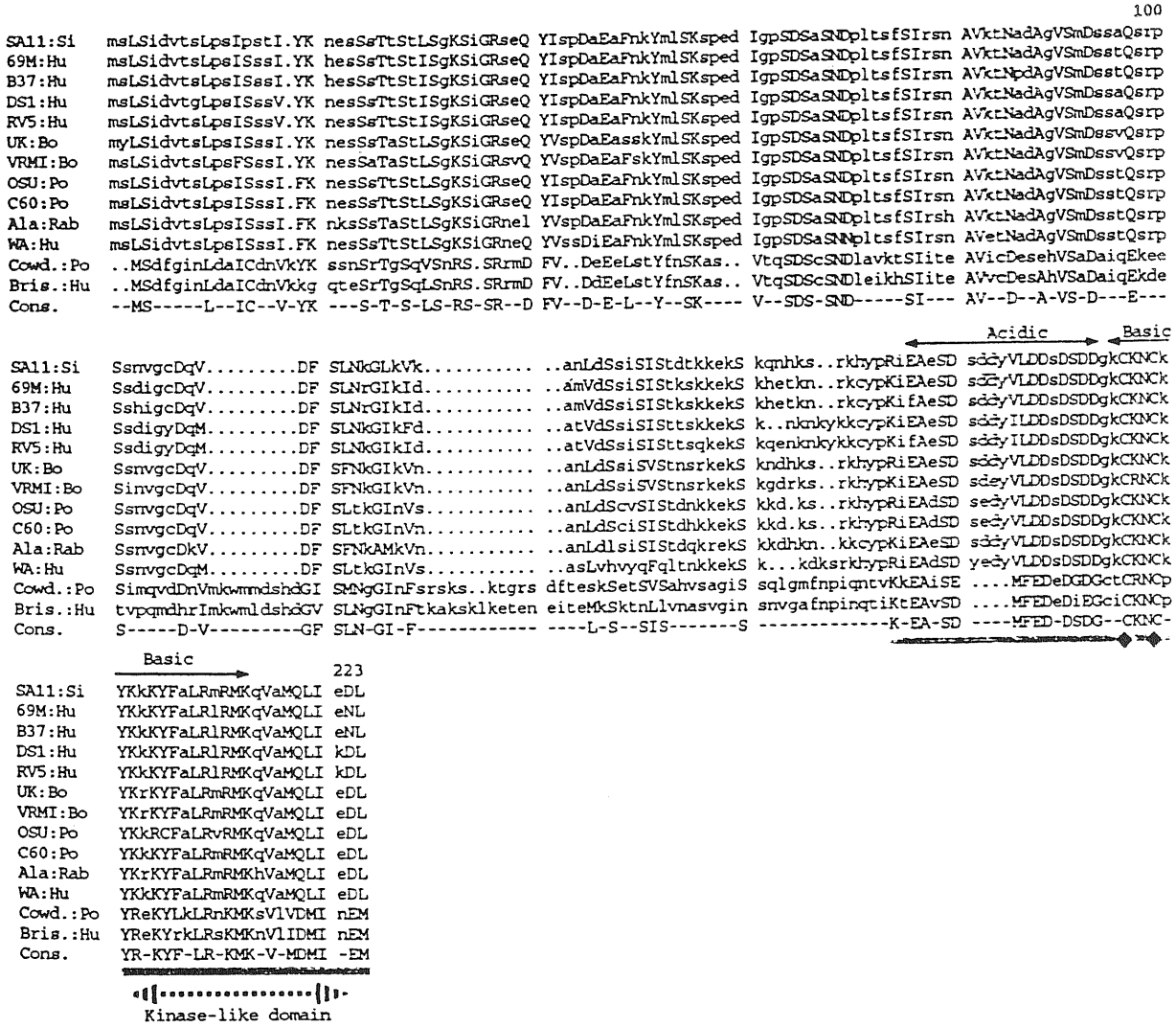
There also are intergroup conserved regions among NSP5 of the three groups. The group A NSP5 shows two basic and one acidic regions at the carboxyl-terminal portion (192). The group A NSP5 carboxyl terminal domain is 100% conserved in all group A rotaviruses. Moreover, the terminal 39 aa of group A NSP5 and group C NSP5 are 38% identical. These two proteins also have carboxyl-terminal basic and acidic domains which in both proteins are separated by a C-X_2-C motif, where X is any aa. This motif in the case of group C NSP5 is C-X-C-X_2-C. The first Cys is not conserved in the group A protein.

In the case of group B NSP5, there is almost no homology with either the group A NSP5 or the group C NSP5. Group B has no cysteins. However, there are basic aa clusters (12-31 and 98-106) and acidic aa clusters (32-42 and 66-87) in group B NSP5. A short domain with partial homology (31.5%) between group A NSP5 (aa 152 to 171 for SA11) and group B NSP5 (aa 113 to 131 for ADRV) also can be found. Both stretches of amino acids are acidic.

Homology with the family of guanido kinases found at the C-terminal end of NSP5 and a putative nucleoside triphosphate binding site at the N terminus of the protein have supported the hypothesis that NSP5 is a protein kinase (3). Based on the presence of a cluster of basic amino acids close to the C-terminus of the protein, it has also been proposed that NSP5 could be an RNA-binding protein (3).

NSP5 has been found localized in the virosomes of virus infected cells where viral replicatio occurs together with other nonstructural protein NSP2 (82) and structural protein VP1, VP2, VP3 and VP6.

Fig 7.



5. THE ROTAVIRUS REPLICATION CYCLE.

5.1 Overview of the Replication Cycle.

Rotavirus replication has been studied primarily in continous cell cultures derived from monkey kidneys, but the natural cell tropism for rotaviruses is the differentiated enterocyte in the small intestine. No direct studies to examine rotavirus replication in intestinal cell lines have been reported. This reflects in part the difficulties to culturing differentiated intestinal cell.

The general features of rotavirus (based on studies in cultures of monkey kidney cells) are as follows:

a. Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This assures activation of viral infectivity by cleaving the outer capsid protein VP4.

b. Replication is totally cytoplasmic.

c. Cells do not contains enzymes to replicate dsRNA, so the virus must supply the necessary enzymes.

d. Transcripts function both to produce proteins and as templates for production of the RNA minus strand. Once the complementary minus strand is synthesized, it remains associated with the plus strand.

e. The dsRNA segments are formed within nascent subviral particles, and free dsRNA or free minus strand ssRNAs are never found in infected cells.

f. subviral particles form in association with virosomes, and these particles mature by budding through the membrane of the ER. In this process particles acquire their outer capsid proteins.

g. Cell lysis releases particles from infected cells.

5.2 Stages of the Replication Cycle:

5.2.1 Adsorption, Penetration, and Uncoating.

The initial stages of virus replication have been examined by biochemical and morphologic procedures. Only triple-layered particles containing VP4 attach to cells when monitored by electron microscopy (EM) (204) or by cell binding (205) or infectivity assays (206). Incresing direct evidence indicates that virus attachment occurs via VP4 (264, 265). Binding to cells does not require cleaved VP4 (207, 208) or glycosylated VP7 (209). It remains possible that the entry steps require both VP4 and VP7.

The identity of the cellular receptor for rotaviruses is not known, but a study of the binding of radiolabeled SA11 to MA104 cells found approximately 13.000 receptor units per cell.

It has been shown (210) that neuraminidase treatment of red blood cells reduces virus binding, indicating a role of sialic acid in virus attachment. Sialic acid-containing compounds such as fetuin and mucin also inhibit virus binding to cell (211, 212). These results add rotaviruses to an increasing number of viruses (such as reoviruses and influenza) that require sialic acid for binding to cells. However, these studies have not determined whether virus binds directly to sialic acid or whether sialic acid maintains the configuraton of the binding site without directly intercating with the virus.

It was demonstrated that binding to sialic acid is not an essential step for animal rotavirus entry into cells (215); thus, sialic acid-resistant mutants were selected and found to retain full infectivity for cells, indicating that there are at least two binding sites on animal rotaviruses. Human rotavirus strains apparently initiate infection of cultured cells by a sialic acid-independent mechanism (213).

Studies of the rotavirus-receptor interaction of a homologous porcine rotavirus with porcine enterocytes have led to the characterization of a cell surface monoganglioside that may function as an *in vivo* relevant receptor for this rotavirus strain, and sialic acid is required for virus binding activity (214). Taken together, all the studies on rotavirus binding and entry suggest that unusual mechanism may be involved. These include binding at two different sites, possibly with two different proteins, and direct membrane penetration of bound viruses rather than conventional receptor-mediated endocytosis. It is possible that rotavirus binding occurs initially by interaction with sialic acid-specific receptors followed by a separate interaction with a second, nonsialyated receptor. Conformational changes in viral binding proteins induced by initial binding effects also may mediate some of the early events.

After binding occurs, virus is internalized. The enchancement of rotavirus infectivity by proteolyses is reportedly due to facilitating the penetration step (207). Internalization will not take place at 0^0 to 4^0C, indicating that this step requires active cellular processes (211, 204). The mechanism of internalization (penetration) into cells is controversial.

Both morphologic and biochemical approaches have been used to investigate the mode of entry of rotaviruses into cells. Early EM studies suggested that virus entry (SA11 strain) occurs by endocytosis (204, 216) and that incoming particles are rapidly transported to lysosomes. Clear documentation of uptake of trypsin-treated virus particles into coated pits, coated vesicles, and secondary lysosomes by EM confirms that rotavirus particles (porcine OSU strain) enter cells by receptor-mediated endocytosis and suggests that uncoating might occur by the effect of lysosomal enzymes (217). Increase the intracellular Ca^{2+} concentration during the early stages of replication was also found to block uncoating (217).These results support early hypothesis that low Ca^{2+} concentration in the intracellular microenvironment may be responsible for uncoating. This data was

originally proposed because it was known that removal of the outer capsid of particles and activation of the endogenous polymerase/transcriptase activity could be accomplished by calcium chelation in a purified virus particle (218, 219).

Studies of the uptake of a human rotavirus whose infectivity is reportedly absolutely dependent on trypsin cleavage suggest that the mode of rotavirus entry into cells differs depending on whether virus particles have been pretreated with trypsin (220). Infectious virus that was pretreated with trypsin was observed to enter cells by direct penetration of particles through the cell membrane into the cell cytoplasm. In contrast, non-trypsin-treated particles were taken up by phagocytosis, and such virions were sequestered into lysosomes 20 minutes after virus attachment to the cell membrane. Further studies are needed to determine whether the common endocytosis-mediated entry pathway exists for rotaviruses as originally thought.

5.2.2 Transcription and Replication.

The synthesis of viral transcripts is mediated by an endogenous viral RNA-dependent RNA polymerase (transcriptase) that has a number of enzymatic activities. The transcriptase is a component of the virion, and properties of this enzyme (or enzyme complex) have been inferred by studing the characteristics of products from *in vitro* transcription reactions (221, 222, 223, 224). Rotavirus particles presumably contain the same enzymatic activities found in reoviruses, including transcriptase, nucleotide phosphohydrolase, guanylyltransferase, and two methylases. These activities are inferred because rotavirus transcripts made *in vitro* in the presence of S-adenosyl methionine posses a methylated 5'-terminal cap structure, m^7GpppGm (225), and transcription is inhibited by pyrophosphate (226).

The virus-associated transcriptase is latent in triple-layered particles and can be activated *in vitro* by treatment with a chalating agent or by heat shock treatment (218, 226). Such treatments result in removal of the outer capsid proteins with conversion of triple-layered particles to double-layered particles (218). In infected cells, triple-layered particles have been shown to be uncoated to double-layered particles, so it is thought that transcription in cells occurs from such particles (227). Transcription is asymmetric, and all transcripts are full-lenght plus strands made off the minus strand of dsRNA (228). The exact site of transcription within the cytoplasm has not been precisely localized, although it is thought to occur also in virosomes.

Activation of transcriptase activity is a process that is not well understood. It has been suggested that this process does not actually modify the enzyme complex but instead releases the templates from structural constraints, allowing them to move past the transcriptase catalytic site (211). Rotavirus transcription requires a hydrolyzable form of ATP, and studies with analogs that inhibit transcription suggest that ATP is required in reactions other than polymerization (226). ATP may be used for initiation or elongation of RNA molecules, as has been described for vesicular stomatitis virus or vaccinia virus RNA polymerases.

The synthesis of plus- and minus-strand RNA has been studied in SA11-infected cells (229) and in a cell-free system using extracts from infected cells (230). Analysis of the kinetics of RNA synthesis in infected cells showed that the synthesis of plus and minus strand RNAs are directed initially at 3 hr postinfection (229). The level of transcription increased until 9 to 12 hr. The maximal level of minus-strand RNA synthesis occurs several hour before the peak of plus-strand RNA synthesis.

The delay in obtaining maximal plus-strand RNA synthesis has been hypothesized to be due to a requirement for the accumulation of stoichiometric amounts of a protein (e.g., VP6) necessary for the assembly of transcriptase particles. Both newly

synthesized and preexisting plus-strand RNA were found to act as templates for minus-strand RNA synthesis throughout infection (230), an unexpected result based on earlier studies with reoviruses (231). The observation that the level of RNA replication does not increase continually in conjunction with the increasing level of plus-strand RNA suggests that RNA replication is regulated by factors other than simply the level of plus-strand RNAs in the infected cells.

Based on nuclease sensitivity in this system, approximately 20% of the RNA made *in vitro* is double stranded and 80% is single stranded. Although not examined directly, it is assumed that rotavirus RNA replication, like that of reovirus, takes place in a conservative fashion; that is, both parental dsRNA strands remain within partially uncoated particles. The synthesis of dsRNA *in vitro* has been determined to be an asymmetrical process in which a nuclease-sensitive plus-strand RNA acts as template for the synthesis of minus-strand RNA. After its synthesis, dsRNA remains associated with subviral particles, suggesting that free dsRNA is not found in cells. Characterization of the subviral particles (complexes separable by sedimentation through sugrose gradient and by equilibrium centrifugation in CsCl gradients) in which dsRNA synthesis occurs both in infected cells and in the cell-free system indicated that these replicase complexes consist of core proteins, VP1 and VP2, small amounts of the VP6, large amounts of the nonstructural protein VP6, large amounts of the nonstructural protein NSP3, and lesser amounts of NSP1 and NSP2 (232). These types of results indicate that some of the NSPs (NSP2, NSP3, NSP5) might be involved in the RNA replication process based on:

a. the presence of these NSPs in replication complexes isolated from infected cells (233, 234).

b. the nucleic acid binding properties of some of the NSPs (NSP2 and NSP3) (235, 236, 237, 238, 239, 240).

c. localization of some NSPs (NSP2 and NSP5) to virosomes (241, 242).

d. the RNA-negative phenotype of ts mutants mapped to the genes that encode these NSPs (266, 267).

A model was hypothesized in which rotavirus double-layered particles were assembled by the sequential addition of VP2 and VP6 to a precore replication intermediate consisting of VP1, VP3, NSP2, NSP3, and NSP5 (243). Because of the inability to completely separate particles with transcriptase and replicase activities with these methods and problems of contamination of some fractions with proteins from neighboring fractions, it has remained unclear whether the components characterized in this system were necessary for RNA replication or simply present in these complexes for other reasons.

The role of individual proteins and specific protein complexes in RNA replication and viral morphogenesis will probably not be solved until they are studied *in vitro* with pure species of native rotavirus proteins and viral RNAs. Progress toward this goal has come from the unexpected discovery that a template-dependent replication system can be established using only viral core proteins and exogenously added purified mRNAs (244). Viruslike particles expressed and self-assembled in insect cells also possess replicase activity (244). The conservation of exogenous mRNA to dsRNA by subviral particles is an exciting result. These results suggest that a cell-free system to support rotavirus RNA replication, transcription, and the assembly of subviral particles could be established (244).

This *in vitro* template-dependent replication system was suprising because it does not absolutely require the nonstructural proteins for replicase activity (244). However, the nonstructural proteins may play a role in increasing the efficiency of replication or play other roles in the replication cycle. This latter idea is supported by the demonstration that NSP1 and NSP3 show a diffuse cytoplasmic staining in cells and NSP3 fractionates with cellular cytoskeletal elements rather than concentrating in

viroplasms (241, 245, 238). NSP3 also binds specifically to a consensus sequence on the 3'end of each of the 11 mRNAs (246). NSP3 may function to stabilize the viral mRNAs from their site of synthesis to the viroplasms. The NSPs may function to recruit or move other viral proteins from their sites of synthesis into the viroplasms, or they may facilitate movement of mascent viral particles from the viroplasms to the ER membrane. Clearly much remains to be learned about the NSPs and RNA binding, transport, replication, and assembly. It is anticipated that these studies will be of general interest because the rotaviruses contain a larger number of NSPs than some of the other viruses within the Reoviridae.

5.2.3 Assembly.

The distinctive feature of rotavirus morphogenesis is that subviral particles, which assemble in cytoplasmic virosomes, bud through the membrane of the ER, and maturing particles are transiently enveloped. This is one of the more interestig aspects of rotavirus replicative cycle, differing from members of other genera in the Reoviridae family. The envelope acquired in this process appears to be lost as particles move toward the interior of the ER, and it is replaced by a thin layer of protein (VP7 and spikes of VP4) that ultimately comprises the outer capsid of mature virions.

Most of the rotavirus structural proteins and all of the nonstructural proteins are synthesized on free ribosomes, although the nascent proteins on free ribosomes have not been analyzed. Instead, this conclusion has been drown based on the absence of signal sequences that would indicate targeting to the ER and lack of protection from digestion in *in vitro* protease protection studies (247, 248). In contrast , the glycoproteins VP7 and NSP4 are synthesized on ribosomes associated with the membrane of the ER and are

cotranslationally inserted into the ER membrane due to signal sequences at their amino termini.

The sites and precise details of RNA replication remain unclear. However, electron-dense viroplasms are probably the site of synthesis of the double-layered particles that contain RNA (242, 265). This conclusion is based on the localization of several of the viral proteins (VP2, NSP2, NSP5) to viroplasms and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER (242), and on the observation that particles emerging from these viroplasms often seem to directly bud into the ER that contains VP7 and NSP4.

Rotavirus maturation reportedly is a calcium-dependent process, based on the observation that virus yields are decreased when produced in cells maintained in calcium-dependent medium (250). Viruses produced in the absence of calcium were found to be exclusively double-layered, and budding of virus particles into the ER was not observed (251). Among the viral proteins, reduced level of VP7 were observed, and sebsequent studies showed that such reduced levels were due to the preferential degradation, and not to the impaired synthesis, of VP7 (251). An interesting finding of these studies was that unglycosylated (but not mature) VP7 made in the presence of tunicamycin was relatively stable in a calcium-free environment. It is possible that calcium stabilizes or modulates folding or compartmentalization of the newly glycosylated VP7 for subsequent assambly into particles. VP7 expresed alone does not fold properly unless it is expressed with other rotavirus proteins, and calcium must be present in cells for correct epitope formation (252, 253). Alternatively, calcium deprivation may destabilize the ER or ER proteins required for the stable association of glycosylated VP7 with the membrane. Outer capsid assembly also requires proper formation of disulfide bonds on VP7 (253).

Understanding viral morphogenesis has been facilitated by the expression of the rotavirus structural proteins individually or in combinations in insects cells using

recombinant baculoviruses (245, 255, 256, 257). This approach first showed that the single-layered VP2 particle shell self-assembles when VP2 is expressed alone, and all of the other capsid proteins have been shown to self assemble into virus-like particles when coexpressed in the proper combinations. Viruslike particles composed of VP2, VP1/2, VP1/2/3, VP2/3, VP2/6, VP2/6/7, VP2/4/6/7, and VP1/2/3/6 have been made and characterised (254, 257). The outer and inner capsid proteins of different virus strains also have been shown to reassociate and be able to be transcapsidated onto other virus strains (268, 269). These results demonstrated that the structural proteins contain the intrinsic information required to form particles and that coexpression of mutant proteins will be a feasible approach to analyse the domains responsible for the structural interactions between the proteins comprising the virus particles.

5.2.4 Virus Release.

EM studies have shown that the infectious cycle ends when progeny virus is released by host cell lysis (260, 261, 262). Despite cell lysis, most double-layered and many triple-layered particles remain associated with the cellular debris, suggesting that these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested (263). Whether particles are simply trapped by the cytoskeleton or the cytoskeleton provides means of transport of viral proteins and particles to discrete sites in the cell for assambly or acts as a stabilizing element at the assembly site and in the newly budded virions remains to be determined.

6. GENERAL FEATURES OF PROTEIN PHOSPHORYLATION AND O-GLYCOSYLATION.

The phosphorylation state of proteins is the net result of the opposing action of two types of enzyme, the protein kinases and the phosphoprotein phosphatases.

Protein kinases have the general property of catalysing the transfer of the γ-phosphate of a nucleoside triphosphate to a hydroxylamino acid residues of a protein substrate. The nucleoside triphosphate is generally ATP, although in a few cases, both ATP and GTP can be phosphate donors (359). The hydroxyamino acceptor can be either serine, serine or threonine, or tyrosine, depending on the particular protein kinase. The main characteristic (other than amino acid sequence) that differentiate individual protein kinases are their physiological substrate and regulatory effectors.

The first protein kinase to be isolated was phosphorylase kinase which is involved in the regulation of glycogen methabolosm (312). During the last 30 years, many protein kinases have been purified. They can be classified as serine/threonine protein kinases and tyrosine protein kinases. Some workers have also reportred the presence of histidine and lysine protein kinases in mammals (329) but nothing is known about the structure of these enzymes.

Ser/Thr protein kinases are often distinguished by their mode of regulation, e.g. cyclic nucleotide-dependent (cAMP or cGMP), calmodolin-dependent diacylglycerol and calcium-regulated protein kinase C (331).

Tyrosine protein kinses were first identified as products of retroviral oncogenes (332). Tyrosine protein kinses are also growth factor receptors for EGF, PDGF, insulin, etc., which become activated in response to ligand binding (370).

All this protein kinases can themselves be regulated positively or negatively by phosphorylation-autologous or heterologous, i.e. by other kinases. Another common

feature of all Ser/Thr and Tyr protein kinases is their sequence similarity in the catalytic domain which allows prediction of where any protein sequence may be a protein kinase (331). The recognition sites for a number of protein kinases have been examined (371). While acidic residues are often located near the Tyr phosphate, the basic residue(s) is often a common site of recognition for cAMP-dependent protein kinase in a motif Arg-X-Ser or Lys-X-Ser and proline seems required for Ser and Thr phosphorylation by the growth-associated protein kinase in a motif Ser-Pro-X-Lys or Thr-Pro-X-Lys (372).

6.1 Substrate specificity of protein kinases.

For some protein kinases, the physiological substrates are unknown, whereas for others they are too numerous to list (363). More precise characterization is often provided using model peptides as substrates (364) and some general conclusions regarding the substrate specificities of certain protein kinases are presented in Table 1. In other cases, however, comparison of the phosphorylated sites of peptides with those of the physiological protein substrate suggests that the tertiary structure of the protein substrate may have a more dominant influence on site specificity. An important corollary of the site specificity of protein kinases is that different phosphorylated residues on a single protein may result from the action of different protein kinases (365).

Table 1. Site specificity of selected protein kinases.

Protein kinase	Specificity	Notes
cAMP-dependent protein kinase	Arg-Arg-U-**Ser#**	U=hydrophobic
Protein kinase C	(Arg/Lys)n-X_m-**Ser#** or **Ser#**-X_m-(Arg/Lys)n	n=2 or 3 m=0 or 1
dsRNA-dependent eIF-2 kinase	Ser-Glu-Leu-**Ser**-Arg-Arg	Specific phosphorylation site in eIF-2
Casein kinase I	Glu_n+-X_m-**Ser**§	n>2, m=0 or 1
Casein kinase II	**Ser#**-(Glu_n+, X_m)	n+m=5, n>3 Acidic residue essential at +3
Tyrosine kinases	(Glu_n+, X_m)-**Tyr**	n+m=4, n>1 Best substrates have several acidic residues but some peptide substrates lack any
Alphaherpesvirus protein kinase	Arg_n-X_2-**Ser#**-Z	n>2 Z not Glu or Pro Studies with model peptides only.

Target amino acids are written in bold.

Thr can replace Ser as the target amino acid.

+ Asp or phosphoserin also possible.

§ (Glu_n, X_m) indicates that, unless otherwisw stated, the positions of Glu and X residues are not specified within a group of n+m residues.

6.2 Autophosphorylation.

Another, apparently general, feature of protein kinases is their ability to undergo autophosphorylation what is usually (but not invariably) an intramolecular reaction. In a virological context, the apparent self-labelling of purified proteins by [g-^{32}P]ATP has frequently been interpreted as autophosphorylation of a protein kinase.

The functional significance of autophosphorylation is a question stil requiring clarification. In some cases there is evidence that autophosphorylation activates the protein kinases, and Krebs (366) has suggested that this may involve displacement of pseudo-substrate sequence, which, in its unphosphorylated form, maintains the enzyme in an inactive state. It should also be mentioned that autophosphorylation frequently occurs in several residues, some of which may be entirely fortuitous and have no effect on catalytic activity.

6.3 O-glycosylation and phosphorylation reciprocity.

6.3.1 O-GlcNAc protein modification.

O-GlcNAc is a simple, monosaccharide moiety glycosidically linked to the side-chain hydroxyls of serine or threonine, often occuring at multiple sites on the same protein (373). Virtually all O-GlcNAc is found within the nucleoplasmic and cytoplasmic compartments of cells (374). O-GlcNAc appears to be highly dynamic and responsive to cellular stimuli in a fashion analogous to phosphorylation.

O-GlcNAc glycosylation is found in all eukaryotes from yeast to man. O-glycosylated is also the adenovirus fiber protein (375). The tegument basic phosphoprotein (UL 32) is the major O-GlcNAc-bearing protein of human cytomegaloviruses (376). The

NSP5 of human rotaviruses (187) and the tegument protein of the baculovirus itself have also been shown to be modified by O-GlcNAc (377). The types of proteins modified in viruses suggest that O-GlcNAc plays a role in assembly reactions of multimeric protein complexes.

6.3.2 O-GlcNAcylation sites are similar or identical to kinase sites.

While about half of the attachment sites for O-GlcNAc occur at or near a "PVS" type motif, about half of the sites have no obvious "consensus" sequence (378). Many of these sites are similar to those used by kinases, particularly glycogen synthase kinase 3 (379), and the MAP kinases such as erk2 kinase (380). Site mapping is allowing site-directed mutagenesis approches to be carried-out in order to evaluate functions. However, if mutation of a specific serine or threonine residue affects a protein functions, it may be difficult to determine if the loss of function is due to inability to attach O-GlcNAc or to attach O-phosphate. Recently, the major site of O-GlcNAcylation of the c-myc oncogene protein was mapped to the transactivation domain (Chou et al., 1995b) at Tyr-58 (381), which is also a major phosphorylation site.

The p53 tumor suppressor was shown to bear O-GlcNAcylated species of p53. Recent studies have also shown that SV40 Large T antigen is O-GlcNAcylated (382). Several O-GlcNAcylated DNA-binding proteins have also been described in the filamentous fungus, Aspergillus orzae, suggesting that O-GlcNAcylation may be important in the functions of transcription factors (383).

6.3.3 O-GlcNAc is a regulatory modification that can be dynamically reciprocal with phosphorylation.

The location of O-GlcNAc at Ser (Thr) sites that are similar or identical to those used by many kinases, and the presence of the modification on many of the cell's most important proteins suggest that O-GlcNAc might be a regulatory modification analogous to phosphorylation. Studies of the activation of murine lymphocytes by mitogens and antigens demonstrated that O-GlcNAc levels on lymphocyte proteins change within minutes after stimulation of the cells (384). Pulse-chase studies on cytokeratines (385) and on the small heat shock proteins (386) clearly show that O-GlcNAc turns over many times faster than the polypeptide to which it is attached. These studies, and those that show the reciprocal nature of Ser(Thr)-O-phosphorylation and Ser(Thr)-O-GlcNAcylation (387) suggest that posttranslational modification of many nuclear and cytoplasmic proteins is a ternary process not a binary one. Thus, the modulation of some protein's functions may be more complicated than the simple addition and removal of phosphate. Rather, it is clear that some proteins exist in up to three diferent isoforms, naked polypeptide, phosphorylated polypeptide and O-GlcNAcylated polypeptide, each with distinct physical and biological properties. Thus far, circumstantial evidence suggests the hypothesis that O-GlcNAcylation of proteins generally favours their associations with other proteins, whereas phosphorylation often appears to favor disassociation.

7. SIGNIFICANCE OF THE PHOSPHORYLATION OF VIRAL PROTEINS.

7.1. Occurence.

When cells infected with a wide variety of viruses are incubated with [P^{32}]orthophosphate, certain viral proteins become radioactively labelled. The animal viruses for which phosphoproteins have been reported include members of the following classes: herpesviridae (196), adenoviridae (403), papovaviridae (258), hepadnaviridae (259), iridoviridae (411), poxviridae (412), African swine fever virus (413), baculoviridae (414), reoviridae (388), parvoviridae (389), picornaviridae (390), togaviridae (391), coronaviridae (392), rhabdoviridae (393), paramyxoviridae (394), orthomyxoviridae (395) and retroviridae (396). In most of he cases, the phosphoproteins identified were structural ones, although to some extend this may reflect the greatest case of detection of the latter. One of the exceptions to this generation is HSV-1, in which case of about a dozen virus-encoded phosphoproteins detected (398, 399), the majority were non-structural. HSV-1 phosphoproteins for which functional activities are known include trans-acting proteins (400), DNase (401), the large subunit of ribonucleotide reductase (402) and the trans-inducing factor that is a structural component of the tegument (397).

7.2 Functional properties of viral phosphoproteins.

7.2.1 Assembly of the virions.

There is evidences that phosphorylation of many viral protein is necessary for their interaction with viral nucleic acid and for subsequent assembly of the virion.

Autographa californica nuclear polyhedrosis virus (AcNPV) pp31 is a nuclear phosphoprotein that accumulates in the virogenic stroma, which is the viral replication center in the infected-cell nucleus, bind to DNA, and serves as a late expression factor (51).

The virion basic phosphoprotein (BPP), UL 32, of the human cytomegalovirus (HCMV) is a 149K tegument protein that represents about 15% of the virion proteins mass and is modified by O-linked N-acetylglucosamine (O-GlcNAc). O-GlcNAc has been postulated to mediate subunit-subunit interaction in many different types of intracellular protein complexes, while BPP may play a role in viral assembly (24).

The nucleoprotein (N), the phosphoprotein (P), and the 22K protein of human respiratory syncytial virus (HRSV) are components of the cytoplasmic inclusion bodies observed in HEp-2-infected cells. Coexpression of N and P was sufficient to induce the formation of N-P complexes detectable by either coimmunoprecipitation with anti-P antibodies or generation of cytoplasmic inclusions (25). Also the assembly of the measles virus nucleoprotein into nucleocapsid-like particles is modulated by its level of phosphorylation (50).

The m1 protein is a major component of the reovirus outer capsid. In the virion outer capsid most m1 exists in the form of a 72K fragment, m1C, that is generated from m1 by proteolytic cleavage (3). Evidence is presented for the existence of one or more phosphoserine residues per molecule of m1C in virions (3).

7.2.2 The role of viral phosphoproteins in viral regulatory processes.

Rex protein from leukemia virus type I is a phosphoprotein which is required for the control of viral structural protein expression and virus replication (52). IE2 is another nuclear phosphoprotein of 80K encoded by the human cytomegalovirus (HCMV) major immediate-early (MIE) gene. It behaves both as a nonspecific transactivator of heterologous reporter genes and as a specific repressor of its own promotor-enhancer region (55).

The gene 61 protein is present in VZV-infected cell nuclei as a heterologous phospho-protein of Mr 62K to 65K. The pp61 exhibits limited aa sequence similarity to the HSV1 nuclear phosphoprotein Vmw 110, which function as a transcriptional factor (57).

The E1 protein of bovine papillomavirus type 1 (BPV-1) is a phosphoprotein which specifically binds and unwinds the virus replication origin by ATP-dependent helicase activity. The E1 protein has been shown to be multiply phosphorylated *in vivo* , although the sites of modification are incompletely mapped (61). As wel the ability of simian virus 40 (SV40) large T antigen to catalyze the initiation of viral DNA replication is regulated by its phosphorylation state (62).

nsP3 protein of Sindbis virus is a phosphoprotein which participate in the synthesis of viral minus-strand and subgenomic RNAs (63). Also in paramyxovirus the large protein (L) and phosphoprotein (P) are both required for viral RNA-dependent RNA polymerase activity (410).

The equine herpesvirus 1 (EHV-1) immediate-early (IE) phosphoprotein is essential for the activation of transcription from viral early and late promotors and regulates transcription from its own promotor (67). In other case the complex between the

nucleocapsid protein (N) and phosphoprotein (P) from vesicular stomatitis virus regulates transcription and a cellular factor(s) in combination with N-P complex may switch the RNA polymerase from transcription to replication mode (68). As well in rabies virus phosphoprotein (P) and nucleoprotein (N) are involved in transcription and replication of the viral genome (68).

The influenza A virus nucleoprotein (NP) is a phosphoprotein that encapsidates the viral genomic RNA. The possible roles of NP phosphorylation for the viral replicative cycle is suggested (69).

Some phosphoproteins have highly antigenic and immunogenic properties as a 30K phosphoprotein (p30) of African Swine Fever Virus (ASFV) is a highly antigenic protein with putative phosphorylation, glycosylation, and membrane attachment sites (81) or the 85K phosphoprotein of human herpesvirus 7 may be considered a major determinant of the human immune response to HHV-7, discriminating HHV-6 from HHV-7 infection (195).

Many phosphoproteins suffer complex regulation of their phosphoriylation or they by themselves participate in complex regulatory mechanisms. For example one of the Hepatitis C virus proteins is a phosphoprotein, and the degree of phosphorylation is regulated by another viral protein. This regulation of phosphorylation may play an important role in modulating the proliferation of virus-infected cells (87). Another viral protein as the UL34 gene product of herpes simplex virus (HSV) is a membrane protein exclusively phosphorylated by the U_S 3 protein kinase which can either directly or indirectly form complexes with several other phosphoproteins (403). The UL37 ORF from the same virus encodes a 120K late (gamma 1) phosphoprotein in infected cells which is part of the tegument structure (193). Polypeptide 4 (HSV-1 ICP4) is a multifunctional phosphoprotein that is essential for viral infection. It is both a repressor and an activator of viral gene expression depending upon the promoter (194). Adenovirus DNA polymerase

(AdPol) is a phosphoprotein and the major *in vivo* phosphorylation site, serine 67, occurs within the consensus substrate recognition sequence for cdc2 kinases. Serine 67 is also one of the major *in vitro* phosphorylation sites, and the substitution of alanine for serine at this position abolishes DNA replication initiation activity of AdPol (70).

8. PROTEIN KINASES ENCODED BY VIRUSES.

Having considered some of the more widely studied viral phosphoproteins, I now turn to the question of protein kinases responsible for the phosphorylation during virus infection.

8.1 Bacteriophage T7 Protein Kinase.

This enzyme was first detected in extracts of infected cells and characterized on the bases of its ability to use ATP to phosphorylate seryl residues in artifitial substrates, such as lysozyme or mixed histones (270, 271, 272). The enzyme was purified and found to be a monomer of M_r 37 000.

Initial evidence for a virion oringe for the protein kinase came from the fact that its induction was prevented by UV irradiation of phage but not by irradiation of the host cells (270) and that protein kinase activity was induced *in vitro* when T7 DNA was used as a template in a coupled transcription/translation system (272). The enzyme was assigned to early gene 0.7 from the fact that viable mutants with deletions in this gene (273) failed to induce the activity (270).

The amino acid sequence of the bacteriophage protein kinase predicted from nucleotide sequence of gene 0.7 (274) shows no apparent similarity to that of any cellular

protein kinase yet described. This includes the E.coli protein-histidine/aspartate kinases (275), the E. coli isocitrate dehydrogenase serine kinase/phosphatase (276) and the eukatyotic family of serine/threonine and tyrosine kinases.

By site-directed mutagenesis of gene 0.7 has been proven that N-terminal domain of the protein contains the protein kinase activity (277). Although conserved in a related bacteriophage (277, 278, 279, 280), the function of the protein kinase activity of gene 0.7 appears more that of "helper"-only important in adverse condition (281) This failure to identify a clear function for the protein kinase domain of gene 0.7 has made it difficult to determine the functionally important substrate of the bacteriophage T7 protein kinase among several proteins dependent on the 0.7 gene for phosphorylation (277).

8.2 Protein Kinases Transduced by Retroviruses.

It is well known that certain retroviruses encode protein kinases that have transforming properties and the importance of the concept of the oncogene resulting from the discovery of such viruses is difficult to overestimate. The confusion probably comes about because the wild-type retroviruses from which they are derived also have transforming properties. Those acutely transforming retroviruses carrying protein kinase genes have transduced the additianal gene from the host cells and their properties result from this being either mutated or abnormally expressed in such a way as to cause celular transformation (282).

The other protein kinases discussed in this section are specific, evolutionarry conserved components of the genetic repertoires of their respective viruses.

Protein Kinase Genes Transduced by Acute Transforming Retroviruses

Name	aa acceptor	Virus	Function
v-src	Tyr	Rous sarcoma virus	Unknown
v-yes	Tyr	Yamaguchi 73 avians arcoma virus	Unknown
v-fgr	Tyr	Gardner-Rasheed feline sarcoma virus	Unknown
v-abl	Tyr	Adelson murine leukemia virus	Unknown
v-fes/fps	Tyr	Gardner-Arnstein and Snyder-Theilen feline sarcoma virus/Fuijinami and PRCII avian sarcoma virus	Unknown
v-erb B receptor	Tyr	Avian erythroblastoma	EGF virus
v-ros	Tyr	U2.avian sarcoma virus	Unknown
v-sea	Tyr	S13 avian erytroleukaemia virus	Unknown
v-fms stimulating receptor.	Tyr	McDonough feline sarcoma virus	Colony factor-1
v-kit	Tyr	Hardy-Zuckerman 4 feline leukaemia virus	Unknown
raf/mil	Ser/Thr	3611 murine sarcoma virus/Mill Hill 2 avian acute leukaemia virus	Unknown
v-mos	Ser/Thr	Moloney murine sarcoma virus	Unknown

Data from Hunter (1991).

8.3 Alphaherpesvirus U_S Protein Kinase.

The first protein kinase gene unequivocally demonstrated in a eukaryotic virus was that encoded in the unique short (U_S) region of the related human and porcine alphaherpesviruses, herpes simplex virus type-1 (HSV-1) and pseudorabies virus (PRV). As this region of the herpesvirus genome is characteristic of the different subclasses of herpesviruses and as the beta and gammaherpesviruses lack a corresponding gene, I shall refer to the enzyme as the alphaherpesvirus protein kinase. It is also known as US3 protein kinase after the designation of its gene in HSV-1. This US3 gene (283) and the corresponding gene US2 or 66 of varicella zoster virus VZV (284), were predicted to encode a protein kinase on the basis of their strong similarity to the family of eukaryotic protein-serine/threonine kinases. Analogous genes have since been described in PRV (285, 286), Marek's disease virus (287) and equine herpesvirus type-1 (EHV-1) (288).

Work characterizing the alphaherpesvirus protein kinase was facilitated by the relative abundance of this enzyme in infected cells and the fact that its substrate-specificity and chromatographic properties distinguished it from cellular protein kinases.

It is now known that other protein kinases are encoded in the herpesvirus genome so it is important to describe the distinguishing properties of the alphaherpesvirus protein kinase. The most well-characterized enzyme, PRV-PK, appears to be a homodimer with a pI of approximately 4.9 and a subunit of 38 kD M_r (289). It catalyzed the transfer of phosphate from ATP (but not GTP) to the seryl or threonyl residues of basic substrates (with a preference for protamine over histone) but not acidic substrates (e.g. casein or phosvitin) and its activity is not dependent on molecules that are able to serve as effectors for the well-characterized cellular protein kinases (290). Further definition of the substrate-specificity has been achived in experiments with artificial peptides (291, 292), RRRRXS/TX being the best artifitial substrate tested. However, peptides with three Arg,

or one more or less "speacer" residue, were also good substrates. This specificity also holds true for HSV-1 PK.

As has been implied above, the US3 gene encoding HSV-1 PK is dispensable for growth of the virus in cell culture (293), although virus lacking US3 has greatly decreased neurovirulence in mice (294). Comparable results have been reported for the analogous gene of HSV-2 (295). It was also possible to disrupt the corresponding gene in PRV, although in this case the mutant grew less well in cultured cells than did the wild-type virus (296). These results clearly exclude an obligatory role of the protein kinase in the virus life cycle, so that the function of the enzyme is most likely to emerge from identifying physiological substrate(s). Experiments with mutant HSV-1 lacking a functional US3 gene (297) have resulted in the identification of the signal physiological substrate known to date: UL34, which is a virion membrane protein of otherwise unknown function (298). Aminoacid residues 191-199 of protein UL34 are predicted to have the sequence RRRRTRRSRE, which was predicted to be a target site for HSV-1 PK (291). Site-directed mutagenesis was used to replace the Thr and Ser residues in this region by Ala and in each case an alteration in the electrophoretic mobility of UL34 indicated decreased phosphorylation (297). It seems unlikely, however, that protein UL 34 is the functionally important substrate of the alphaherpesvirus protein kinase. This is because in the two other alphaherpesviruses for which sequences are available, the highly homologous protein 24 of VZV (299) and protein 26 of EHV-1 (300) completely lack a counterpart to this phosphorylation site. It should perhapes be emphasized at this point that, although HSV-1 PK and PRV-PK have been shown immunocemically to be present in the virion (in which cellular protein kinases are also to be found (301)), most of their activity appears to be in the soluble fraction of the infected cell (302).

Alphaherpesvirus protein kinases clearly constitute a separate family, although-presumably because of the rate of evolution of viruses-they are more diverged from one

another than are families of cellular protein kinases. One can say that they have no obvious cellular homologue: indeed, the CDC2 kinase, which gives one of the best scores in pairwise comparison (303), is quite distant from them.

8.4 Herpesvirus U_L Protein Kinase.

The product of the gene corresponding to the virion protein, UL13, of HSV-1 (304) might also be a protein kinase related to the cellular protein-serine/threonine kinase family (305, 306).

This family of proteins is evolutionary more distant from the cellular protein kinases than are the alphaherpesvirus U_S protein kinases. The protein kinases in this family range from 429-707 aa in lenght. Most of the non-catalytic region is at the N-terminus, and, unlike the situation with the alphaherpesvirus U_S protein kinases, there is some discernable sequence similarity here. This similarity lies within a region of approximately 70 amino acids on the N-terminal side of the start of the catalytic domain.

Evidence has been obtained supporting the prediction that gene UL13 and its equivalents-at least in the alphaherpesviruses-do indeed encode a protein kinase. De Wind et al. (307) performed experiments in which the UL13-equivalent of PRV was transiently expressed in eukaryotic cells and the product immunoprecipitated. The immunoprecipitate catalyzed the phosphorylation of seryl residues in casein and histones (although not protamine, the best artifitial substrate for the alphaherpesvirus U_S protein kinase) and also appears to perform autophosphorylation.

Other workers have focussed on the protein kinase activities of infected cells. Preparations of a protein kinase isolated from the nuclei of cells infected with HSV-1, although by no means pure, contained and had the ability to phosphorylate, UL13 (304). (Similar observations have been made with virions (309)). Clearly this might be

autophosphorylation. As with the transiently expressed PRV kinase, casein but not protamine served as an endogenous artifitial substrate. The activity was able to use both GTP and ATP as a phosphate donor, a property of the ubiquitous cellular CKII. However, it differed from the cellular enyme in not been inhibited by heparine.

Less is knownn of the function of the herpesvirus U_L protein kinase. Like the alphaherpesvirus U_S protein kinase, its gene has been shown to be dispensable for growth in cell culture in the case of both HSV-1 (308) and PRV (307). The mutant of HSV-1 lacking gene UL13 fails to phosphorylate the regulatory protein a22 (the product of gene US1), suggesting that this latter may be a substrate of the protein kinase (308). Perhapes the phosphorylation plays a role in regulating viral transcription.

8.5 Channel Catfish Virus Protein Kinases.

Channel catfish virus (CCV) has been assigned to the herpesvirus family mainly on the basis of its morphology. The recently determined DNA sequence of its 134 Kb genome provides only weak support for this assignation: certainly if it is a herpesvirus it is the most diverged member of the family known (310). CCV appears to possess no equivalent of either the U_S or U_L protein kinases of other herpesviruses, but does not contain two families of related genes (ORFs 73 and 74 and ORFs 14, 15 and 16) the predicted product of which show homology to the cellular eukaryotic protein kinase family (311, 310).

8.6 Poxvirus and Related Protein Kinases.

The orthopox viruses have an larger genome than do herpesviruses and the determination of the DNA sequence of the prototype vaccinia, revealed a gene, B1, with

the potential of encoding a protein with strong homology to the eukaryotic cellular protein kinase family (313, 314).

B1 is the only putative gene product of vaccinia virus that is strongly related to the cellular protein kinases (315), but it, in turn, is related to another vaccinia protein, the product of gene B12. Most of the conserved catalytic residues of protein kinases that are present in the product of B1 have equivalents in the productof B12, the Asp-166 of the cAMPdPK is replaced by Lys (315). From the preceding consideration of the catalytic function of this residue, one would not, therefore, expect B12 to exhibit protein kinase activity. It can not be excluded that the B12 gene product, although lacking protein kinase activity itself, might modulate the properties of the B1 protein kinase *in vivo* .

At least two temperature-sensitive mutants in gene B1 have been described that can arrest infection of mouse L-cells at the stage of replication in viral DNA (313). This does not, of course, imply a direct function of the protein kinase in DNA replication, but does indicate a more central role in the vaccinia replicative cycle than is the case for the herpesvirus protein kinases. There have been reports of several phosphorylated proteins during vaccinia virus infection, including virin structural protein VP11 (317, 318), the cellular ribosomal proteins S2, Sa and S13 (319, 320, 321) and the major viral DNA-binding protein (323).

The other protein kinase to be mentioned in this section is one encoded by African swine fever virus. African swine fever virus has a morphology similar to iridoviruses, but its genome shares some similarities with that of the poxviruses. The sequence of gene L191L would predict that it encodes a 244 aa protein kinase and this has been shown to be the case by expression of recombinant protein in bacterial cells (324).

8.7 Baculovirus Protein Kinase.

The baculovirus, *Aytographa californica* , is a large double-stranded DNA virus that infects insect cells. It has been used to construct vectors for the expression of recombinant eucaryotic proteins, including protein kinases and it is, therefore, important to draw attention to a recent but little publicized observation that suggests that the virus itself encodes a protein kinase.

The sequence of a 9.4 Kb segment of the genome of thi virus (333) included that of an ORF9 predicted to encode a protein of 196 aa. Posse at al. (333) did not comment on the possible function of this gene, but computer analysis suggests that it is related to cellular protein kinases (334). This putative protein kinase seems to be most closely related to cellular protein kinases that are activated by calcium, but its approximately 250 aa residues would appear insufficient to induce a calcium-binding domain.

8.8 Protein Kinase Activity Associated with HSV Ribonucleotide Reductase.

Herpesviruses analysed to date contain distinct genes for the two different subunits of a ribonucleotide reductase homologous to that of eukaryotic and procaryotic cells. The large subunit of the ribonucleotide reductase of HSV-1 and HSV-2 differs from most other cellular and virus equivalents in possessing an additional N-terminal domain that is not required for ribonucleotide reductase activity (325). This region of the genome of HSV-2 has attracted considerable interest as it lies in the BamHI E fragment, which has transforming properties. There is no direct evidence that the transforming properties of the BamHI E fragment are a consequence of an encoded protein kinase activity. This would

require a correlation between the associated kinase activity and transforming ability in the sort of inactivating point mutants. To date, the only studies of this type appear to be with a relatively large fragment of HSV-2 encoding aa 72-502 (335). Finally it should be remarked that this region of the HSV-1 genome does not have transforming properties (336), so that if the N-terminal portion of the R1 of both HSV-1 and HSV-2 should encode a protein kinase, it would be difficult to see how it could account for the unique transforming properties of the HSV-2 fragment.

Aurelian and co-workers were the first to decribe a protein kinase activity associated with the large subunit of ribonucleotide reductase (R1) from HSV-2, but (in their hands) not with that from HSV-1 (326). Although weak incorporation into histones was reported, most of their work has used the property of the protein kinase activity to phosphorylate the large subunit itself. In their original paper they drew attention to what they regarded as a similarity between regions of the N-terminal domain of R1 and some of the conserved motifs of eukaryotic cellular protein kinases and it is the proposal that these features in the N-terminal domain could be responsible for the protein kinase activity (presumed to be autophosphorylation).

8.9 Protein Kinase Activity of Hepatitis B Virus Hbx Protein.

It was concluded in the previuos section that if the large subunit of HSV ribonucleotide reductase contains a protein kinase activity this latter must differ from the other viral protein kinase so far discussed in being unrelated to the main family of eukariotic cellular protein kinases. There is strong evidence for such an unorthodox viral protein kinase activity. The hbx protein of hepatitis virus, which has a M_r of 16,500 only half the size of the eukaryotic protein kinase domain, to which , in any case, it shows no homology. The hbx protein is a rather general trans-activator of transcription and is an

essential viral protein, as might be expected for a virus with such a small genome (3.2 kb). The claim that this protein possess an autophosphorylating protein kinase activity is based on the fact that activity was found in protein expressed and purified from a plasmid vector in E.coli, translated from mRNA in reticulocyte lysate and immunoprecipitated, or separated by electrophoresis and transferred to a nitrocellulose membrane (337). Furthermore, an ATP-affinity analogue concomitantly inhibited both protein kinase and trans-activating activities. These results make it unlikely that the protein kinase activity is a contaminant, although it will be easier to exclude this possibility when the physiological substrate is identified and can be used to assay protein kinase activity. Both hbx and hbx-phosphorylating protein kinase activity were detected in virions (337) and it is possible that this activity corresponds to a virion protein kinase activity reported previously (338, 339, 340).

8.10 Protein Kinase Activity of VSV L-proteins.

Transcription of RNA templates from VSV *in vitro* requires the viral L protein (which has RNA polymerase activity) and the more abundent NS protein (now more generally reffered to as the P protein). Two phosphorylated species of NS/P, NS1 and NS2, are found *in vitro*, both in the cytoplasm of infected cells and in the virion, although the virion cores contain only NS1, the less extensively phosphorylated of the two species (341). RNA transcription *in vitro* with an RNA template-N protein complex and the L and NS/P proteins only proceeds if the most extensively phosphorylated species of NS/P, NS2, is used (354) and the activity of NS2 can be abolished by treatment with alkaline phosphatase (355). A similar functional importance has been established for the phosphorylation of the analogous NP protein of influenza virus (342).

Benerjee and coworkers (343, 344) have proposed that the phosphorylation of the NS/P protein is catalysed by the L protein, which is large enough (M_r 241,000) to be multifunctional (indeed, it also possess methyltransferase activity (356), although it does not contain a domain homologous to the eukaryotic cellular protein kinase family. Purified preparations of L protein do have an associated protein kinase ativity (345) that can catalyse the phosphorylation of NS, although it appears to prefer NS2 rather than NS1 as substrate (346). Because the protein kinase ativity associated with L protein has a substrate-specificity similar to that of CKII, the caution was required before accepting that this activity was intrinsic rather than a contaminant (357). However, it has now been shown that phosphorylation of NS/P by cellular CKII must occur before that catalyzed by the kinase activity associated with the L protein and that the two activities can be clearly distiguished (347). There have also been reports from other groups supporting the contention that the VSV (358) and related Sendai virus (348) L proteins have intrinsic protein kinase activity, although, contradicting these, it has been reported that the kinase activity can be separated from the L protein (349). Certainly, the idea of the association of protein kinase activity with transcription factors has gained respectability (350), but it will require the identification by mutagenesis of functional residues before it be concluded that the L protein contains an intrinsic protein kinase activity.

CHAPTER 2

METHODS

1. Cells and Viruses.

MA104 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine, 50 mg/ml gentamycin (Gibco Laboratories). Rotavirus simian SA11 and porcine Osu strains were propagated and grown in MA104 cells for almost two days when the complete virus cytopatic effect was observed (407). The inoculums were colected and centrifuge at low speed to remove cellular debrees. The supernatants were recovered and conserved at -80°C. C7-MA cell line was obtained by transfecting MA104 cells with pKG4-11 plasmid containig the complete coding region of the Osu rotavirus NSP5 (408) under the control of the SV40 promoter region. Transfectants were selected by resistance to 500 mg/ml G418 (Geneticin, Gibco Laboratories).

2. Liposome mediated transient transfections of adherent MA104 cells.

MA104 cells were transiently transfected/cotransfected by lipofectin (GibcoBRL, Life Technologies) or transfectam (Promega) with any of the deletion mutants, the wild type NSP5 and/or NSP2 genes cloned in KpnI/EcoRV polylinker site of pCMV eucaryotic vector under T7 promotor. The genes were expressed by vTF7 expression system.

Confluent monolayers of MA104 cells in 30 mm diameter Petri dish were infected with vaccinia (vTF7) at multiplicity (20 PFU of trypsin-activated vaccinia per cell). After 30 min to 1 h the virus inoculum was removed and the cells were washed twice

with DMEM serum-free medium. Later on the cells were transfected with 2 µg plasmid DNA (Qiagen or GetStar purified) for single transfection and 4 µg DNA in the case of cotransfection experiments. DNA for each transfection was diluted in 100 µl (for lipofectin transfection) or 250 µl (for transfectam transfection) DMEM medium w/o serum. As well 15 µl lipofectin reagent or 5µl transfectam reagent was diluted into 100 µl or 250 µl, respectively, serum-free DMEM medium and allowed to stand at room temperature for 30-45 min, when lipofectin was used only. DNA-solution and Lipofectin or Transfectam solution were combined, mix gently, and incubated at room temperature for 10-15 min. For each transfection to each tube containing the Lipofectin/Transfectam reagent-DNA complexes was added 0.8 µl serum-free DMEM medium, mixed gently and overlayed the complex onto the cells. The cells were incubated for 12 to 15 h at 37^0C in a CO_2 incubator. At this time post-transfection the DNA-containing medium was removed. The cells were washed two times with PBS and cellular extracts were prepared in 50 µl TNN lysis buffer at 4^oC. Extracts were spun at 10 000 g for 7 min and supernatants used for western immunoblot analysis.

3. Western immunoblot analysis.

Lysates of infected and mock infected MA104 cells or transfected cells (corresponding to $0.5x10^5$ cells) were resolved on a (SDS)-12% polyacrylamide gels (409). Proteins were transferred onto a nitro-cellulose (Sartorius) or PVDF (Millipore) membrane and reacted to a guinea pig anti-NSP5 serum. As a second antibody was used anti-ginea pig antibody-HRP conjugated (DAKO immunoglobulins).

4. Radiolabeling of rotavirus-infected cells with [^{35}S]-methionine, [^{32}P]P_i and (1,6)-[^{3}H]glucosamine.

Confluent monolayers of MA104 cells in 60 mm diameter petri dishes were infected at high multiplicity (20-30 PFU of trypsin-activated SA11 Rotavirus per cell). At 3-4 hr post-infection cells were incubated with methionine-free RPMI medium containing 50 mCi/ml (1000 Ci/mmol) of [^{35}S]methionine (Amersham). For pulse-chase experiments, the cells were labeled for 15 min in methionine-deficient RPMI medium containing 50 mCi/ml of [^{35}S]methionine. The labeling medium was removed and replaced with RPMI medium supplemented with 0.25 mg/ml methionine and further incubated for 2 hr. For ^{32}P labeling, cells were fed with 2 ml phosphate-free minimal essential medium for 30 min at 2.5 hr post-infection and labeled by adding 600 mCi of [^{32}P]P_i (Amersham) for 2 hr at 37°C. Labeling with [1,6-^{3}H]glucosamine (52 Ci/mmol; New England Nuclear) was performed essentially as described (187): after viral adsorption, cells were washed twice with phosphate-buffered saline (PBS) and incubated in glucose-deficient DMEM medium containing 0.1 mg glucose/ml; at 4 hr postinfection cells were washed again with PBS and incubated in glucose-free DMEM containing 5 mg AD/ml and 200 mCi [1,6-^{3}H]glucosamine (52 Ci/mmol; New England Nuclear) for 2 hr. After labeling cells were washed and lysed in 100ml TNN lysis buffer (100mM Tris.HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet-P40, 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C. Extracts were spun at 10,000 *g* for 7 min and supernatants used for immunoprecipitation analysis.

5. Chemical crosslinking of rotavirus infected and mock infected cells.

Confluent monolayers of MA104 infected and mock infected cells in 60 mm diameter petri dishes were labeled with ^{35}S methionine or ^{32}P as previously described in (4). The labeling medium was removed and the cells were washed twice with PBS containing 1mM Mg^{2+} and 1mM Ca^{2+}. The cells were overlayed with 1.5 ml washing buffer and DSP chemical crosslinker (Pierce) was added to the cells in 600 mM final concentration. The cells were incubated at 4°C for 15 min. The crosslinker was quenched twice with 50 mM Tris pH 7.5, 150 mM NaCl buffer for 3 min each time at 4°C. The quenching buffer was removed and total cellular extracts were prepared in 100 µl TNN. Extracts were spun at 10 000 *g* for 7 min and supernatants used for immunoprecipitation analysis.

6. UV-crosslinking of rotavirus infected or mock infected cells.

Confluent monolayers of MA104 infected and mock infected cells in 60 mm diameter petri dishes were labeled with ^{35}S methionine as previously described. The labeling medium was removed and the cells were washed twice with PBS. The cells were overlayed with RIPA buffer and UV crosslinking was performed in UV Stratalinker™ 1800 for 3 min (486 000 mJ) on ice. The distance between the cells monolayer and the UV lamp was kept 22 sm. The extracts were prepared in RIPA buffer. Extracts were spun at 10 000 *g* for 7 min and supernatants used for immunoprecipitation analysis.

7. Immunoprecipitations and PAGE analysis.

10 to 20 µl of ^{35}S- and ^{32}P- labeled cellular lysates crosslinked or noncrosslinked were diluted up to 100 µl with TNN buffer and subjected to immunoprecipitation by incubation with 1 ml anti-NSP5 or anti-NSP2 serum at 4°C during 2 hr. A 40 µl aliquot of 50% Protein-A Sepharose CL-4B beads (Pharmacia) in TNN buffer was added and further incubated for 60 min at 4°C. Beads were washed three times with RIPA buffer (50 mM Tris.HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS). Antigen-antibody complexes were released by boiling in 4x Laemmli's sample buffer and analysed by electrophoresis on (SDS)- 12% PAGE. Visualization of ^{35}S- and ^{3}H-labeled proteins were enhanced by fluorography using Amplify (Amersham). Autoradiography was performed at -70°C using X-ray film (Kodak X-OMAT AR).

Note: The crosslinked extracts were pretreated with 1 µl anti-mouse preimmune serum for 1 h at 4°C in 100 ml TNN and 40 ml 50% Protein A Sepharose CL-4B beads (Pharmacia). The supernatants were recovered and immunoprecipitaed with anti-NSP5 or anti-NSP2 as it is described.

8. Sequential immunoprecipitation.

100 ml supernatants from crosslinked and noncrosslinked pretreated extracts as described in (7-note) were subjected to immunoprecipitation by incubation with 1 µl anti-NSP2 serum at 4°C during 1 hr. A 40 ml aliquot of 50% Protein-A Sepharose CL-4B beads (Pharmacia) was added and the incubation was continued for another 1 h at 4°C. The beads were washed and analysed as it is described in (7). The supernatant was

collected and the immunoprecipitation with anti-NSP2 repeated. The beads were washed again and analysed as it is described in (7) and the supernatant was collected and immunoprecipitated with anti-NSP5 serum. The beads were proceed as in the previous times and the antigen-antibody complexes were released by boiling in 4x Laemmli's sample buffer and analysed by electrophoresis on (SDS)- 12% PAGE.

9. Phosphatase treatments.

For calf intestinal alkaline phosphatase (CIP) (New England Biolabs) treatments, immunoprecipitates were resuspended in 20 µl reaction buffer containing 10 mM Tris HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 1 ml CIP (10 000 U/ml) and incubated at 37°C for 1 to 3 hr. Lambda protein phosphatase (λ-PPase) (New England Biolabs) treatment was performed by resuspending the immunoprecipitates in 40 ml reaction buffer containing 50 mM Tris.HCl pH7.8, 5 mM DTT, 2 mM $MnCl_2$, 100 mg/ml BSA and 1 ml λ-PPase (400 000 U/ml) and incubating at 30°C for 1 hr.

When the total cellular extracts from transfected cells had to be analysed and treated with λ-PPase the reactions were performed in following way: 3 ml total extracts was incubated with 3 µl l-PPase (400 000 U/ml) in 25 ml reaction buffer containing 50 mM Tris HCl, pH 7.8, 5 mM DTT, 2 mM $MnCl_2$, 100 mg/ml BSA at 30°C for 2 h.

10. *In vitro* phosphorylation.

Sepharose beads containing immunoprecipitates were washed twice with RIPA buffer and once with kinase buffer (50 mM Tris.HCl, pH 8.0, 1.5 mM spermidine,

5 mM $MgCl_2$, 1 mM DTT, 5% glycerol).The reaction was carried out in 20 ml kinase buffer containing 10 mCi (3000 Ci/mmol) [γ-^{32}P]ATP (Amersham) for 30 min at 37°C. Unincorporated [γ-^{32}P]ATP was removed by three washes with RIPA buffer before protein elution. The immunoprecipitates were eluted by 4xLaemli's Sample buffer containing 40% glycerol, 5% β-mercaptoethanol, 6% SDS, 125 mM Tris pH 6.8, 0.04% Bromphenol blue at 95°C for 3 min. The supernatants were analysed on 12% PAGE.

11. Phosphoamino acids analysis.

Phosphoamino acids were analysed by the method of Cooper et al. (363). *In vivo* and *in vitro* ^{32}P-labeled NSP5 was immunoprecipiatedand resuspended in 5.7 N HCl (Merck). Protein-A Sepharose beads were pelleted by centrifugation and the supernatant was hydrolysed for 2 hr at 110°C. Hydrolysates were dried under vacuum and dissolved in pH 1.9 buffer (44.4:156:1800, 99% formic acid: glacial acetic acid: H_20) containing 1 mg each of phosphoserine, phosphothreonine and phosphotyrosine as standards. Samples were applied to 10 x 20 cm cellulose thin layer plates (Merck) and electrophoresed in pH 1.9 buffer at 1200 V for 25 min After drying, a second dimension of electrophoresis in pH 3.5 buffer (10:100:1890, pyridine: glacial acetic acid:H_2O) was performed at 2000 V for 40 min Plates were dried, sprayed with ninhydrin and exposed to X-ray film for autoradiography.

12. *In vitro* translation.

The protein coding region of the NSP5 cDNA was subcloned downstream of the T3 RNA polymerase promotor in the transcription plasmid pSP6/T3 (BRL) to yield plasmid pT3-NSP5. The construct was transcribed using T3 RNA polymerase (Pharmacia Biotech) and the transcript translated in rabbit reticulocyte lysate (Promega). After 1 hr of translation, extracts of non-infected or infected MA104 cells, or immunoprecipitated NSP5, were added and further incubated for 1 hr at 37°C. Translation products were immunoprecipitated with anti-NSP5 antiserum and subjected to λ-PPase treatment when indicated and analysed in (SDS)-12% PAGE.

13. Production of anti-NSP2 and anti-NSP5 serum

The antigens NSP2 and NSP5 were cloned in frame with a secretion peptide sequence, obtained from Vh region of IgG, under CMV promotor in pcDNA3 to generate plasmids pcDNA3/sp-NSP2 and pcDNA3/sp-NSP5.

The plasmids were purified separately by CsCl gradient or Qiagen protocol. The pDNA for each injection was dissolved in 0.8% NaCl in maximum volume of 100 µl. A single mouse was injected with a mix of pcDNA3-antigen (50 mg) and pcDNA3-mGM-CSF (50 mg) (328). Six/seven weeks Balb/c mice were injected with intradermic puncture at the tail base 1 to 2 sm from the body. Seven days later the injection was repeated. At day 14 was performed the last bust. The first blood extraction was applied at day 42nd and the second and the last at day 56th. The collected serum was kept at 37^0C for 1 h. After that it was centrifuged at 1000 rpm for 5 min. The supernatants was colected and stored

with 0.02% NaN_3 at -20^{0}C. The serum was checked for specificity by westrn blotting analysis against extracts of infected cells.

14. Oligonucleotide primers.

Oligonucleotides were synthesised by the solid phase triester methodology on a DNA synthesizer (380A; Applied Biosystem, Inc., Forster City, CA). Following synthesis, the oligonucleotides were removed from the solid support by an ammonia treatment (55^{O}C) overnight, dried under vacuum, and purified by G-50 Sephadex molecular sieve chromatography using TE elution buffer. These oligonucleotides were futher used as PCR primers.

15. PCR deletion mutagenesis of NSP5.

NSP5 deletion mutants were produced by PCR truncation of the N-terminal and C-terminal part of NSP5 gene with specific primers.

First NSP5 gene with its 5'-and 3'-nontranslated regions was cloned in KpnI/HindIII site of pOsu5/Pst I plasmid. pOsu5/Pst I plasmid was linealized by Xmn I digestion to create a template for PCR reactions. All PCR reactions were performed on the pOsu5/Pst I//Xmn I-digested plasmid in 100 ml reaction mixture containing each dNTP at 250 mM, 15pM of each PCR primer, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus) and PCR Reaction Buffer provided by the same manufacturer.

C-terminal truncations were performed using as a forword primer always 16-mer 5' AACAGCTATGACCATG 3' which is complementary to 5'-upstream portion of

NSP5 gene. For reversed amplification were used primers as follows: 5' TTACTGCAGAGTTGAGATTGATAC 3' (complementary to the aa at positions 125-130); 5' TTACTGCAGAATTCTCGGGTAGT 3' (comlementary to the aa at positions 145-149); 5' TTACTGCAGACCGTCATCACTAT 3' (comlementary to the aa at positions 163-168); and 5' TTACTGCAGGTACTTTTTC 3' (complementary to the aa at positions 175-179) which corespond respectively to ΔC67, ΔC48, ΔC29 and ΔC18 deletion mutants. Any of these reverse primers contains in its 5' end portion Pst I restriction site followed by a stop codon.

N-terminal truncations were performed using as a forword primers; 5' AATGGTACCATGATTGGTAGGAG 3' (complementary to the aa at positions 33-37) and 5' AATGGTACCATGGTTAAGACAAATG 3' (complementary to the aa at positions 80-84) which correspond respectively to ΔN33 and ΔN80 deletion mutants. Any of these forword primers contains in its 5' end portion Kpn I restriction site followed by ATG initiation codon for methionine. As a reversed primer was used 17-mer 5' GTAAAACGACGGCCAGT 3' which is complementary to the 3'-downstream region of NSP5 gene.

N- and C-terminal double truncation was performed using as a forword primer ΔN33 deletion mutant forword primer and as a reversed one was used ΔC67 deletion mutant reversed primer.

The amplifications were carried on in the following thermal cycler (Perkin Elmer Cetus) conditions: 95^{0}C 1 min, 40^{0}C 2min, and 72^{0}C 2 min, for 6 cycles followed by other 25 cycles at 95^{0}C 1 min, 40^{0}C 1 min, and 72^{0}C 2 min. 10 μl of each PCR product were analysed on 1% agarose gel by its expected size compered to 1 kb DNA ladder (BRL).

16. Cloning and sequencing of the PCR deletion mutants.

The different PCR bands seen on 1% agarose gel and coressponding to the expected size of the respective deletion mutant were purified by electroelution. The recovered fragments were subjected to Kpn I/Pst I digestion again repurified by electroelution and subsequently ligated in Kpn I/Pst I site of pOsu5/Pst I and used to transform E. coli strain DH5a. Clones were picked randomly, and double-stranded DNA template was prepared and sequenced by the dideoxy chain termination method (406) using the T7 Sequencing Kit (Pharmacia LKB).

From pOsu5/Pst I positive clones the deletion mutants were subcloned in Kpn I/Hind III site of pBluescript^{R} K/S (+/-) under T3 promotor to create pT3/ΔC67, pT3/ΔC48, pT3/ΔC29, pT3/ΔC18, pT3/ΔN33, pT3/ΔN80 and pT3/Δ-Δ constructs.

From pBluescript^{R} K/S (+/-) the deletion mutants were subcloned in Kpn I/EcoR V site of pCMV eukaryotic vector under T7 and CMV promotors to create pCMV/ΔC67, pCMV/ΔC48, pCMV/ΔC29, pCMV/ΔC18, pCMV/ΔN33, pCMV/ΔN80 and pCMV/Δ-Δ constructs.

NSP5 and NSP2 wild type sequences from T3/NSP5 and T3/NSP2 vectors also were subcloned in Kpn I/EcoR V site and Bam HI/Xba I site, respectively of pCMV vector to generate pCMV/NSP5 and pCMV/NSP2 constructs.

CHAPTER 3

RESULTS

1. Phosphorylation of rotavirus NSP5.

1.1 Multiple forms of post-translationally modified NSP5.

The rotavirus NSP5 protein produced in virus-infected cells was analyzed by immunoblotting of electrophoresed total cellular extracts (Fig. 8a) or by immunoprecipitation of the [^{35}S]methionine labelled material followed by (SDS)-PAGE (Fig. 8b). As expected, two major bands of apparent molecular mass of 26 and 28 kDa (K) were obtained. However, additional material which appeared as a smear (and sometimes as a double band) with a molecular mass of up to 34K, was also clearly detected in both cases (lanes 1 and 2). A similar pattern of bands was observed when NSP5 was immunoprecipitated from virus-infected cells labelled *in vivo* with $^{32}P_i$ (Fig. 8c). In this case, the 32-34K band became more evident while the 26K protein was less labelled. That these NSP5 forms were the consequence of phosphorylation was demonstrated by treatments of the immunoprecipitates with two different phosphatases, the recombinant bacterial serine/threonine and tyrosine λ protein phosphatase (λ-Ppase) and the alkaline calf intestine phosphatase (CIP). In both cases a main band of 26K was obtained (Fig. 8b and 8c). Although CIP treatment was also efficient in removing phosphates and reducing the size of the 32-34K protein, it was in general less effective, and produced a substantial amount of the 28K protein. The intensity of the 26K band increased when the protein was labelled with [^{35}S]methionine, indicating a complete conversion to the smaller form. On the other hand, as expected, when NSP5 was labelled with ^{32}P most of the label associated with the 28K and 32-34K bands was lost, though a

small amount was retained in the 26K form (Fig. 8c). We have been unable to remove all the labelled phosphate from the 26K band even after prolonged and repeated treatments indicating that some of the phosphorylation sites are resistant to the phosphatases used.

These results indicated that part of the newly synthesized NSP5 in virus infected cells was in a hyperphosphorylated form. This conclusion was in agreement with the results obtained by immunoprecipitations of extracts of infected cells that had been labelled with a pulse of [^{35}S]methionine for 15 minutes and then chased for 2 hr. As shown in Figure 8 (d), a substantial amount of NSP5 of 32-34K accumulated after the chase period (lane 8) while very little was detected immediately after the pulse (lane 7). In spite of this, the 28K form remained as the major product.

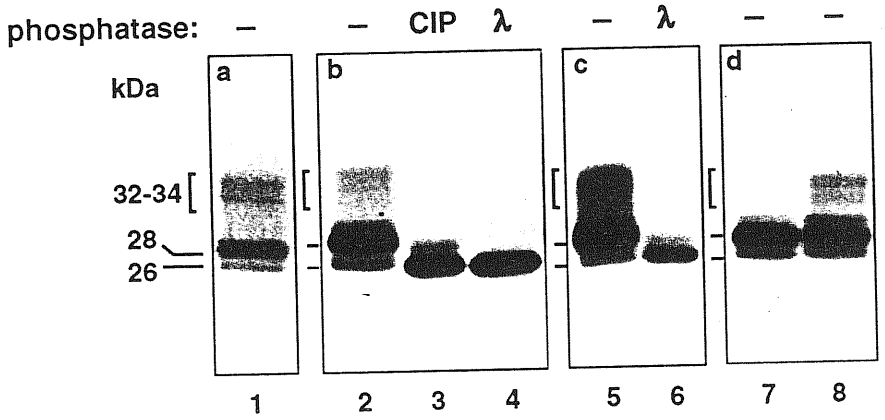


Fig.8. SDS-PAGE analysis of NSP5. (a) Immunoblot analysis of extracts of MA104 cells infected with rotavirus SA11 (4 h post-infection) and reacted with anti-NSP5 serum. (b-d) Immunoprecipitation of NSP5 from virus-infected cells labelled *in vivo* with: (b) [^{35}S]methionine by continuous labelling; (d) [^{35}S]methionine by pulse (lane7) and chase (lane8) labelling; (c) ^{32}Pi, CIP and λ-Ppase treatments are indicated.

1.2 *In vitro* phosphorylation of NSP5.

The results obtained *in vivo* prompted us to investigate the possibility of phosphorylating NSP5 *in vitro*. Figure 9 (a) shows that when immunoprecipitates of NSP5 were incubated solely with [γ-^{32}P]ATP a characteristic pattern of phosphorylation was obtained that differed from the one obtained *in vivo* regarding the relative intensities of the various forms. The major product was represented by the 32-34K form, followed by the 28K form, while that of 26K form was scarcely labelled (lane 1). No phosphorylation was obtained when the immunoprecipitates were derived either from infected cells using an anti-VP4 serum or from non-infected cells using anti-NSP5 antibody (Fig. 9b). NSP5 phosphorylation also took place employing total cellular extracts from virus infected cells instead of immunoprecipitates (data not shown). Treatment of *in vitro* phosphorylated NSP5, with λ-PPase also produced a conversion into the 26K band conserving residual [^{32}P] (lane 6). These results indicated that all the differentially phosphorylated NSP5 forms can still be further phosphorylated *in vitro*.

Figure 10 shows a similar *in vitro* phosphorylation with [γ-^{32}P]ATP, performed with extracts of cells that had been previously labelled with [^{35}S]methionine. The two panels correspond to the same gel exposed to detect ^{35}S and ^{32}P (a) and ^{32}P only (b). The bands of 26, 28 and 32-34K from both panels are completely superimposable. CIP and λ-PPase treatments shifted the label from the 32-34K band towards those of 28 and 26K forms (lanes 2) or completely into the 26K form (lanes 3), respectively.

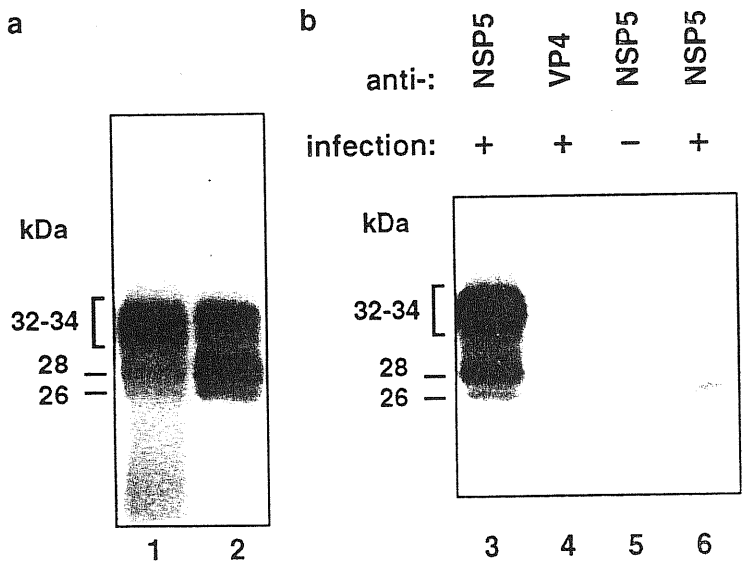


Fig.9. *In vitro* phosphorylation of NSP5. (a) SDS-PAGE of immunoprecipitates of NSP5 phosphorylated *in vitro* with [γ-^{32}P]ATP (lane 1) and, for comparison , of *in vivo* ^{32}P-labelling NSP5 (lane 2). (b) SDS-PAGE of *in vitro* phosphorylation reactions carried out with immunoprecipitates obtained from non infected (lane 5) or virus-infected (lane 3, 4 and 6) MA104 cells using anti-NSP5 (lane 3, 5 and 6) or anti-VP4 (lane4) sera. The sample of lane 6 was treated with λ-PPase.

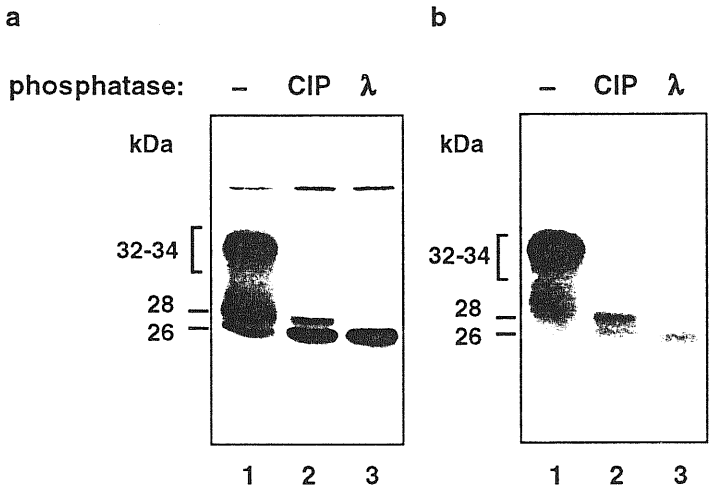


Fig.10. Double labelling of NSP5. SDS-PAGE analysis of *in vivo* [^{35}S]methionine-labelled NSP5 immunoprecipitated and phosphorylated *in vitro* with [γ-^{32}P]ATP (lanes 1). Treatments with CIP and λ-PPase are indicated (lanes 2 and 3). (a) Autoradiogram corresponding to ^{35}S and ^{32}P. (b) Autoradiogram corresponding only to [^{32}P].

1.3 Analysis of phosphorylated residues.

The amino acid residues phosphorylated both *in vivo* and *in vitro* were determined by partial acid hydrolysis followed by two dimensional thin layer electrophoresis of the phosphoamino acids. As shown in Figure 11, only phosphoserine and phosphothreonine were obtained in both cases. This result is not surprising considering the high content of serine and threonine (24.5%) of NSP5.

Since the change in electrophoretic mobility of NSP5 due to phosphorylation was very significant, we also analysed the soluble products obtained after partial alkaline hydrolysis (327) of ^{32}P-labeled NSP5. Only P_i was obtained, thus ruling out the formation of polyphospate chains as a consequence of the process of hyperphosphorylation (results not shown).

1.4 NSP5 phosphorylation in the absence of viral infection.

We also investigated whether other viral proteins were involved in the phosphorylation of NSP5. For this pourpose we derived a stable transfectant of the MA104 cell line (C7-MA104) that constituvely expressed cytoplasmic NSP5. As shown in Figure 12, the [^{35}S]methionine-labelled NSP5 immunoprecipitated from this cell line was composed of the two major 26 and 28K bands (lane 1) while the material of higher relative molecular mass was hardly detected. Nevertheless, *in vivo* ^{32}P-labelled NSP5 from C7-MA104 cells produced all isoforms (lane 3), although their relative proportions were different from those observed in virus-infected cells. Treatment with l-PPase produced, as expected, the 26K band (lanes 2, 4). This result strongly suggests that no other viral protein is required for the post-translational phosphorylation of NSP5.

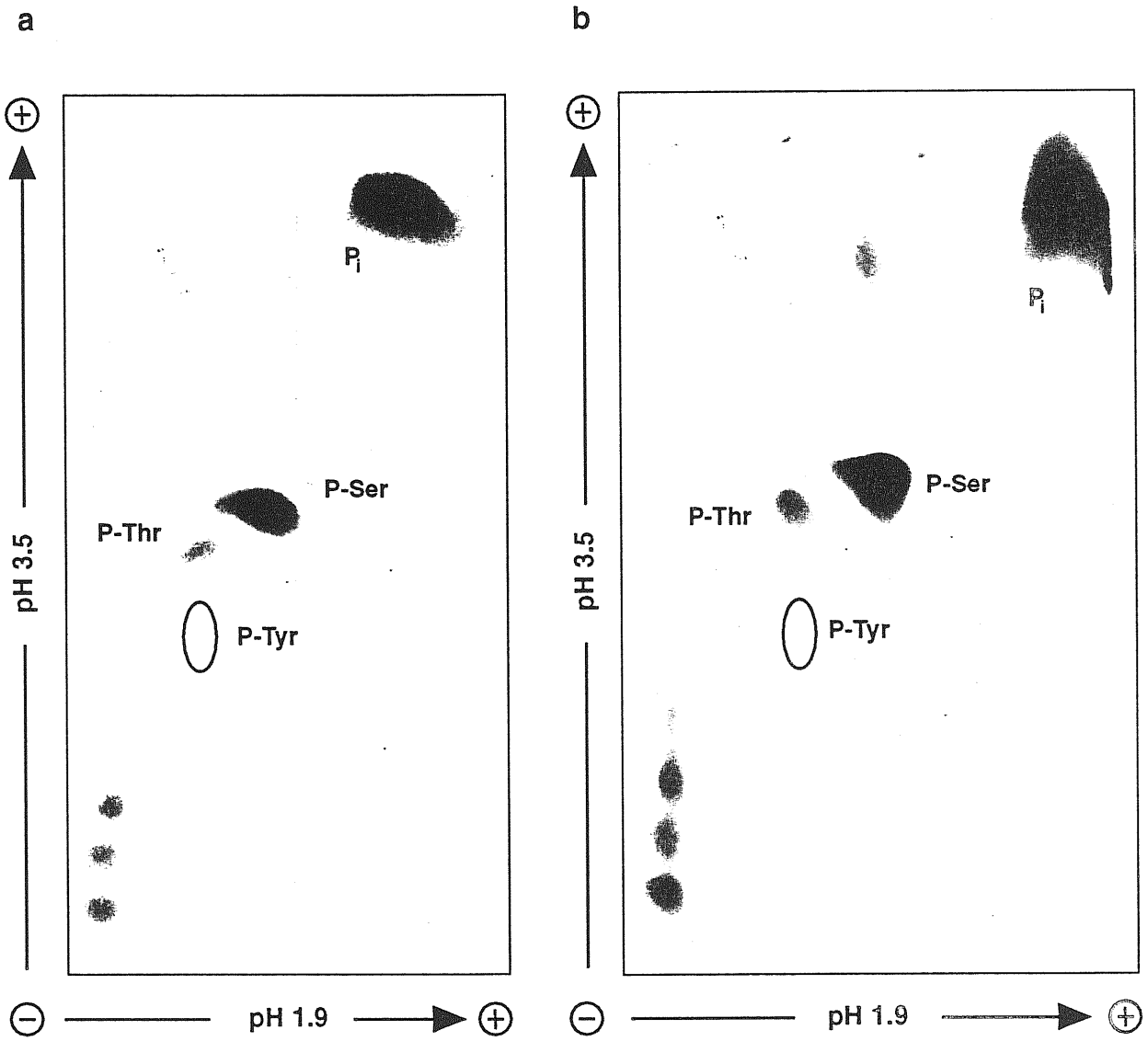


Fig.11. Autoradiograms of the two-dimensional thin-layer electrophoresis corresponding to phosphoaminoacid analysis of *in vivo* (a) and *in vitro* (b) ^{32}P-labelled NSP5.

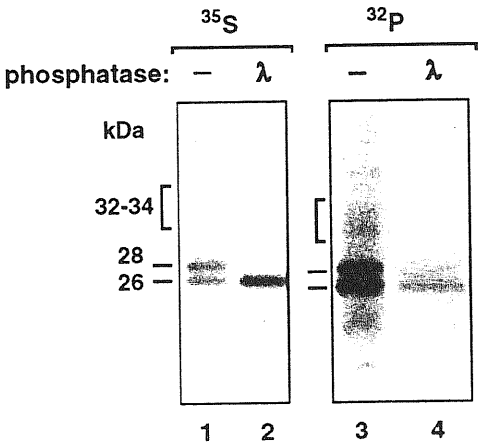


Fig.12. SDS-PAGE analysis of immunoprecipitates of NSP5 from extracts of C7-MA cells, labelled *in vivo* either with [^{35}S]methionine (lanes 1 and 2) or ^{32}Pi (lanes 3 and 4) and treated with λ-PPase as indicated.

1.5 Post-translational modification of *in vitro* translated NSP5.

The product of *in vitro* translated NSP5 mRNA in a reticulocyte lysate system consisted of a precursor molecule with an apparent molecular mass of 25 kDa which was clearly different from the 26K band obtained *in vivo*, even after λ-PPase treatment (Fig. 13, lanes 1, 2, 3). This mobility difference is most likely due to O-linked N-acetylglucosamine residues present in the 26K form (see below). When incubated with an extract of MA104 virus-infected cells, the 25K NSP5 precursor could be converted into a 28 kDa protein, while no effect was observed with extracts of non-infected cells (lanes 4 and 5). The same conversion was also possible by addition of NSP5 immunoprecipitates, instead of total lysates, although with a lower efficiency (not shown). The change from 25K to 28K was demonstrated to be due to phosphorylation by its sensitivity to λ-PPase treatment (lane 6).

1.6 NSP5 O-linked glycosylation and phosphorylation.

We have previously shown that NSP5 is post-translationally modified by the addition of single residues of O-linked N-acetylglucosamine (328). [^{3}H]glucosamine incorporation in virus-infected cells yielded the two NSP5 isoforms of 26 and 28K. The data presented here demonstrated that the changes in the electrophoretic mobility (from 26K to 28K and 32-34K) were due to the addition of phosphates which can be removed by phosphatases. To investigate the O-glycosylation in the various NSP5 isoforms, virus-infected MA104 cells were metabolically labeled with [1,6-^{3}H]glucosamine, and the extracts immunoprecipitated with anti-NSP5 serum. Figure 14 (a) shows that, following a short period labelling (2h), the [^{3}H]glucosamine label was distributed between the 26 and

28K bands with practically no label in the 32-34K forms (lane 2). However, longer labelling period (4h) with [^{3}H]glucosamine showed that some material of molecular mass greater than 28K was produced (Fig. 14b, lane 5). Thus the hyperphosphorylated forms (32-34K) appear to contain very little or none of the O-glycosidic residues. Nevertheless, a single band of 26K of increased intensity was obtained after λ-Ppase treatment (lanes 1 and 6). These results demonstrated that the various *in vivo* forms of NSP5 have different relative contents of O-linked N-acetylglucosamine and phosphates and, in addition, that their differences in electrophoretic mobility were due to the degree of phosphorylation.

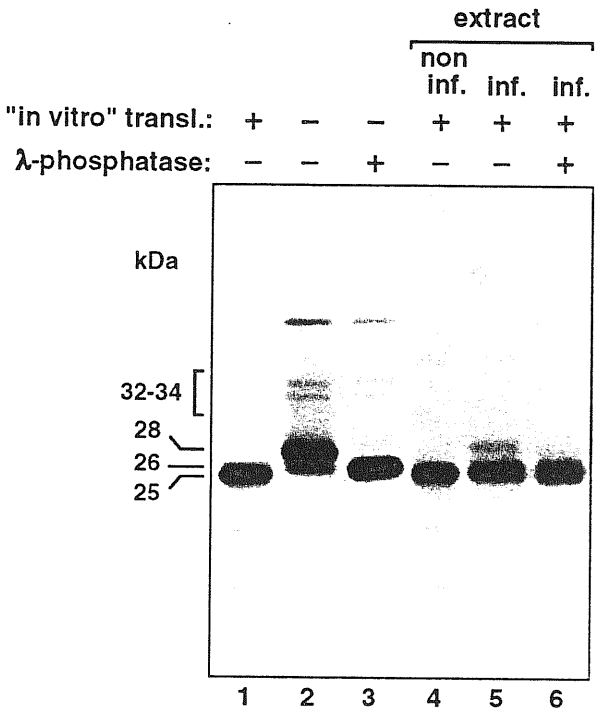


Fig.13. *In vitro* translation of NSP5. SDS-PAGE analysis of immunoprecipitates of NSP5 obtained by *in vitro* translation in a reticulocyte lysate system (lanes 1, 4, 5 and 6), and invubated with extracts of non-infected (lane 4) or virus-infected (lanes 5 and 6) cells. For comparison, immunoprecipitated *in vivo* [^{35}S]methionine-labelled NSP5 (lanes 2 and 3) was included. Samples treated with λ-Ppase are indicated.

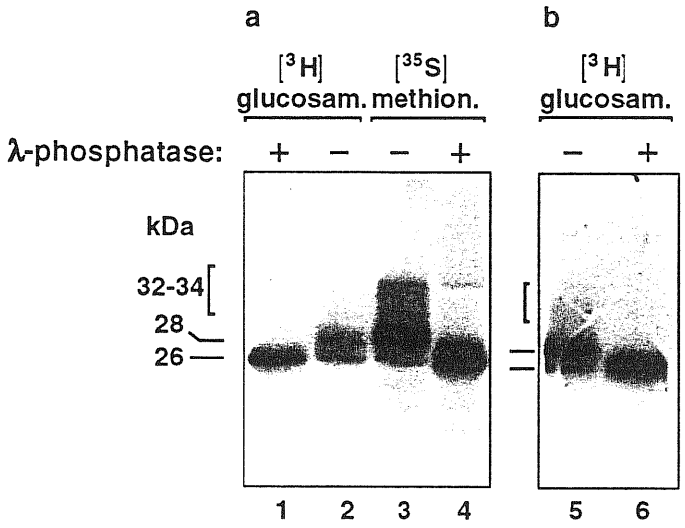


Fig.14. Immunoprecipitation of NSP5 labelled *in vivo* with [1,6-^{3}H]acetylglucosamine for a period of 2 h (a) or 4 h (b). In (a), lanes 3 and 4 correspond to labelling with [^{35}S]methionine. Samples treated with λ-Ppase are indicated.

2. Interaction of the rotavirus NSP5 with other viral proteins.

2.1 *In vivo* crosslinking of MA104 rotavirus infected cells.

To investigate the possible interactions of NSP5 with other proteins we performed *in vivo* chemical (DSP) crosslinking of MA104 rotavirus infected cells as well as MA104 mock infected cells. We chose DSP crosslinker because it is the most widely used one which is cell permeable and thiol cleavable (it can be cleaved by reduction with 5% β-mercaptoethanol in SDS-PAGE sample buffer). In our experiments the cells were labeled for 2 h with [^{35}S]-methionine at 3-4 hr post-infection and then crosslinked by DSP. As a control the same experiment was repeated without DSP-treatment. Total cellular extracts prepared from the crosslinked and non-crosslinked infected and mock infected cells were immunoprecipitated with anti-NSP5 antibody. The immunoprecipitates were analysed on 12%PAGE in reducing (with β-mercaptoethanol) and nonreducing (without β-mercaptoethanol) conditions (Fig. 15). Interestingly, in the immunoprecipitates obtained from the crosslineked extracts, in addition to NSP5 two other proteins were observed. They were localized at positions corresponding to 35K and 120K which were coincident with those of the rotavirus proteins NSP2 and VP1. These two proteins can be easily observed only in total extracts of infected cells. NSP2 and VP1 have not been previously described to interact with NSP5.

When the crosslinked complex was analysed on non-reducing gels we observed a main band at a position of very high molecular weight which corresponded to the crosslinked complex(s) although a faint band at the position of NSP2 was also visible (Fig. 15, b). This residual amount of NSP2 shows that not all of the NSP2 that interacts with NSP5 can be crosslinked.

The finding that the band detected in the position of NSP2 really corresponds to rotavirus NSP2 and is not a hyperphosphorylated form of NSP5, which could appear in the immunoprecipitates from the crosslinked extract, needed further demonstration. For this purpose a mouse polyclonal antibody against NSP2 was produced. When this antibody was used to immunoprecipitate extracts of infected cells the protein observed corresponded to the same band obtained with anti-NSP5 antibody from crosslinked extracts (Fig. 15, c-lane 4 and 5).

λ-Ppase treatment of the immunoprecipitates was another way to confirm that the protein observed above NSP5 on the 12%PAGE was the rotavirus NSP2 and not one of the multiple phosphorylated forms of NSP5. As shown in Figure 15 (c) λ-Ppase treatment of the immunoprecipitates from crosslinked extracts demonstrated complete reduction of the NSP5 32-34K and 28K phosphorylated forms to 26K while no reduction of the 35K band was observed (lane 4). This result was consistent with the fact that the observed protein was NSP2 and not a hyperphosphorylated form of NSP5.

Another approach to look for intermolecular interactions was the use of UV-induced crosslinking. UV-irradiation induces activation of different atoms or groups of a molecule which could react with other reactive sites (425). These interactions are often very stable. The UV-crosslinking protocol is mainly used to detect protein/nucleic acids complexes. Eventhough it is not so efficient for protein/protein interactions we used it as a second approach to study NSP5 interactions.

Figure 16 (a) shows that NSP5/NSP2 interaction can also be detected with this method. In the pulse-chase *in vivo* labeling experiment the conversion of the 26K precursor form of NSP5 to the 32-34K phosphorylated forms was clearly seen in the immunoprecipitates obtained from extracts chased for 2hr after [^{35}S] methionine labelling of MA104 rotavirus infected cells (Fig. 16, a-lane 2). Lane 3 and 4 represent anti-NSP5 immunoprecipitates from extracts of MA104 rotavirus infected cells, following UV-

crosslinking *in vivo*. In the UV-crosslinked extracts NSP5 interacted preferentially with NSP2, while no visible interaction between NSP5 and VP1 was detected. However, the NSP5/NSP2 interaction was clearly visible, thus suggesting that NSP5 interacts distinctly with both proteins. The peculiarities of these interactions will be discussed more in detail in chapter 4.

As in the case of DSP crosslinking, the reduction of NSP5-hyperphosphorylated forms to 26K after λ-PPase treatment was also demonstrated for the UV crosslinked extracts. As expected, since the 35K band represents NSP2, no modification of its positon in the gel was observed after phosphatase treatment.

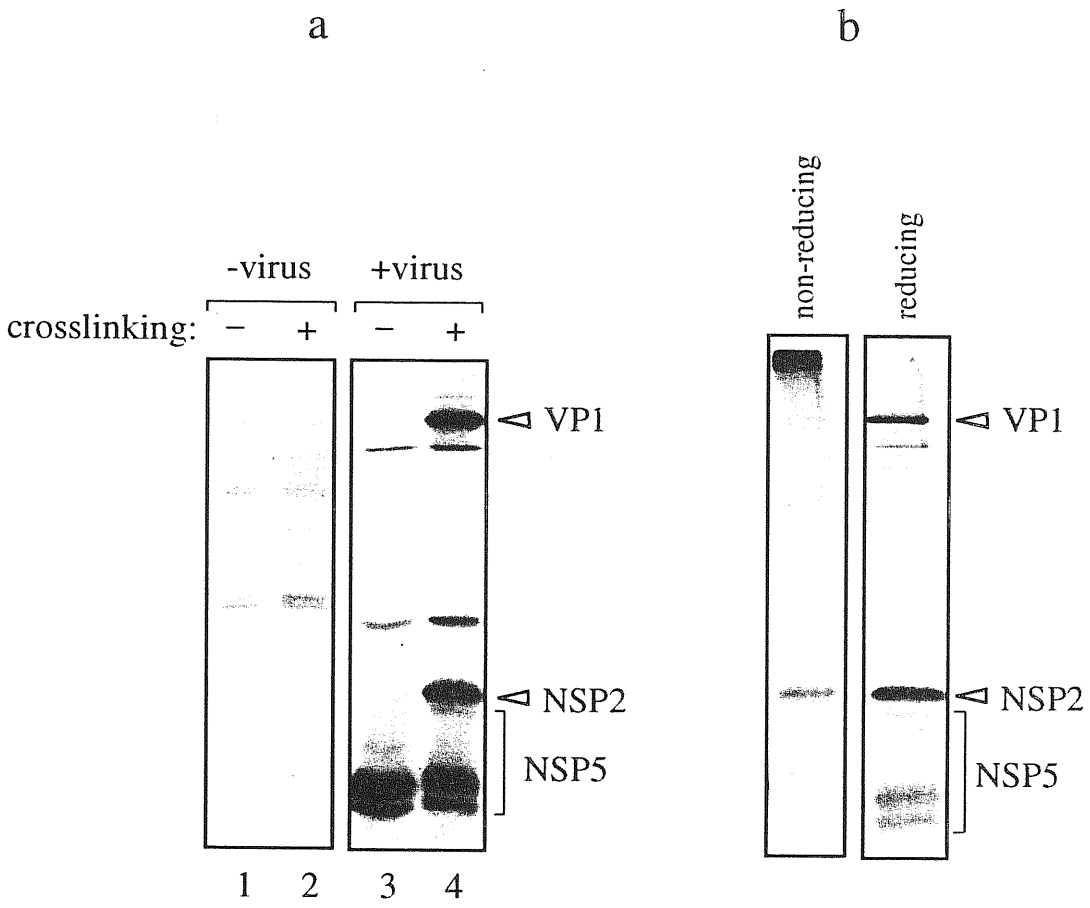
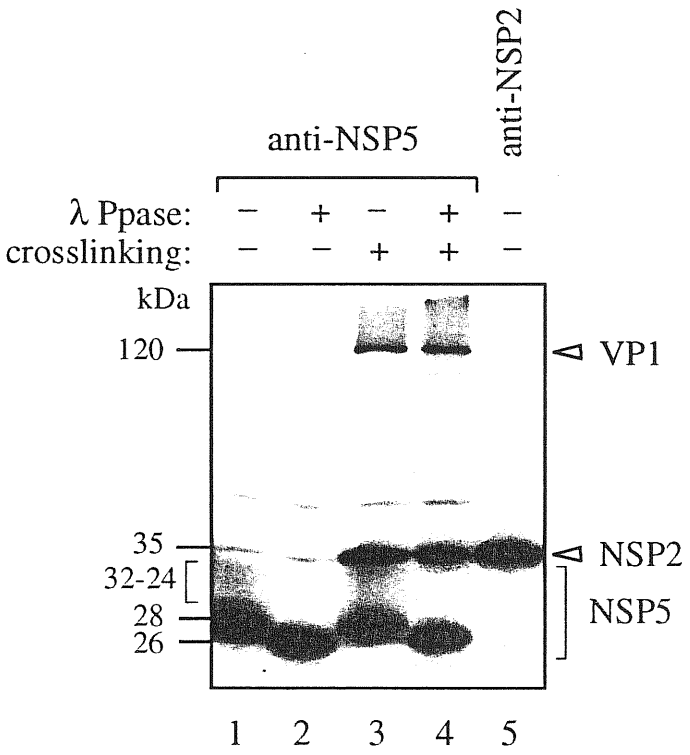


Fig.15. Analysis of DSP-crosslinked extracts from MA104 rotavirus infected cells: (a) reducing SDS-PAGE analysis of anti-NSP5 immunoprecipitates obtained from non-crosslinked and DSP-crosslinked extracts of non-infected and infected MA104 cells; (b) analysis in non-reducing and reducing condition of anti-NSP5 immunoprecipitates obtained from DSP-crosslinked infected cells.



(c) SDS-PAGE analysis of anti NSP5 immunoprecipitates from non-crosslinked and crosslinked extracts of infected cells and treated with λ-PPase as indicated. Lane 5 shows anti-NSP2 immunoprecipitation.

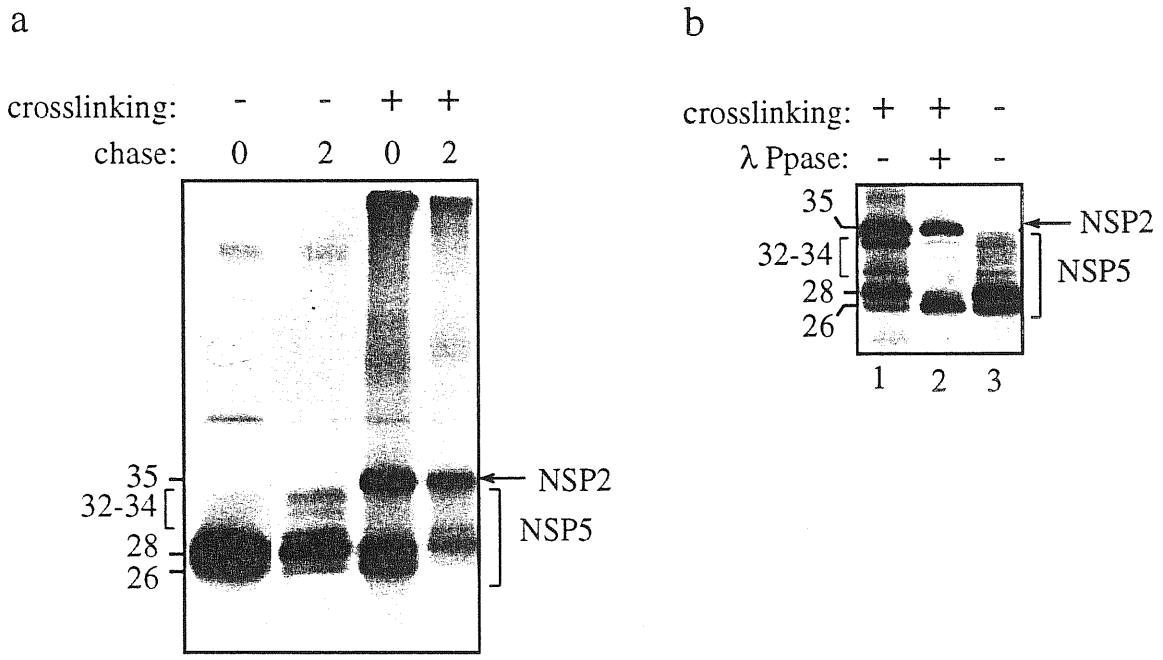


Fig.16. Analysis of anti-NSP5 immunoprecipitates obtained from UV crosslinked extracts of MA104 rotavirus infected cells. (a) Lane 1 and 2 represent anti-NSP5 immunoprecipitates obtained from pulse and chased [^{35}S]methionine labeled extracts of MA104 infected cells, which have been UV crosslinked as indicated. Panel **b** shows a similar experiment in which the crosslinked immunopresipitate was λ-Ppase treated.

2.2 Sequential immunoprecipitaion of crosslinked and non-crosslinked extracts.

To better understand the interaction between NSP2, VP1 and NSP5 we decided to perform sequential immunoprecipitations (Fig. 17). The crosslinked and non-crosslinked extracts were sequentially immunoprecipitated with anti-NSP2 antibody two times followed by anti-NSP5 immunoprecipitation. In Figure 17 the last three lanes show that anti-NSP2 antibody can coprecipitate VP1 although not in large amount and only a small amount of NSP5 from the crosslinked extracts. It is also clear that it still remains a significant amount of NSP2 which is not immunoprecipitated after two cycles of anti-NSP2 that nevertheless is precipitated by anti-NSP5 (lane 6). In addition, the amount of VP1 is also more represented in the crosslinked complex immunoprecipitated by anti-NSP5 than by anti-NSP2. This result indicates that NSP5 interacts mainly with VP1 and less with NSP2 and also that VP1 interacts preferentially with NSP5 and less with NSP2. As expected in non crosslinked extracts anti-NSP2 did not immunoprecipitate neither NSP5 nor VP1.

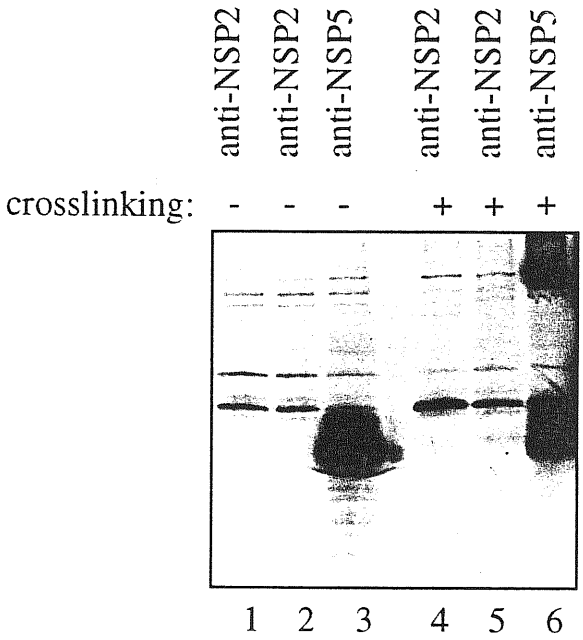


Fig.17. Anti-NSP2 and anti-NSP5 sequential immunoprecipitations: lanes 1 and 2, and 4 and 5, represent two sequential anti-NSP2 immunoprecipitation which where followed by a third immunoprecipitation with anti-NSP5 as indicated. Lane 1-3 non-crosslinked extracts. Lane 4-6 crosslinked extracts.

3. Rotavirus NSP2 upregulates the hyperphosphorylation activity of NSP5 *in vivo* and *in vitro*.

The previous section contains biochemical evidences that NSP5 interacts with NSP2 and the viral polymerase VP1. On the basis of the results obtained we decided to investigate the role of NSP5/NSP2 interaction on NSP5 phosphorylation.

3.1 *In vitro* phosphorylation of NSP5 in the presence of NSP2.

We first analysed the effect of DSP crosslinking on the phosphorylation of NSP5 in the *in vitro* phosphorylation assay. For this purpose, MA 104 rotavirus infected cells were labeled at 3-4 hr postinfection with [^{35}S] methionine for 2 hr. The cells were then subjected to chemical DSP-crosslinking and total cellular extracts immunoprecipitated with anti-NSP5. The results are shown in Figure 18. A characteristic pattern of phosphorylation was observed in the case of the immunoprecipitates obtained from the non-crosslinked extracts. The main phosphorylated form of NSP5 was the 32-34K protein, while scarce phosphorylation was observed in the 28K form (Fig. 18, b-lane 1).

In the immunoprecipitates from the crosslinked extracts there was an increase in the o-phosphate incorporation in the 32-34K band of NSP5 and diminished phosphorylation of the 28K form with respect to what was observed in the non-crosslinked extracts (Fig 18, b-lane 3). Interestingly, although the immunoprecipitates obtained from the crosslinked extracts represented a complex (or complexes) of three proteins NSP2, NSP5 and VP1, neither VP1 nor NSP2 appeared to be phosphorylated in this *in vitro* assay. Treatment with λ-Ppase of the immunoprecipitate from non-crosslinked extract gave the expected pattern. All the phosphorylated material observed

was reduced to 26K form (Fig. 18, a-lane 2). No residual amount of phosphate in the position of the 26K band was observed in panel b which represents the autoradiography of only the ^{32}P radioactivity of panel a. On the other hand, λ-Ppase treatment of the immunoprecipitates from crosslinked extracts of infected cells gave the NSP5 band of 26K while the 35K band of NSP2 was not sensitive to λ-Ppase (Fig. 18, a-lane 4). The residual amount of phosphate observed in panel b (lane 4) suggests the existence of different phosphorylation sites in NSP5, some of them not recognized by λ-Ppase.

That NSP2 was not phosphorylated *in vivo* in virus infected cells was also demonstrated in the experiment shown in Figure 19. Indeed, when the MA104 infected cells were labelled with ^{32}P and the immunoprecipitates with anti-NSP5 and anti-NSP2 resolved on PAGE, NSP5 showed the characteristic phosphorylation pattern while no phosphorylation was detected in the anti-NSP2 immunoprecipitates. This result indicates that NSP2 does not undergo phosphorylation during viral replication.

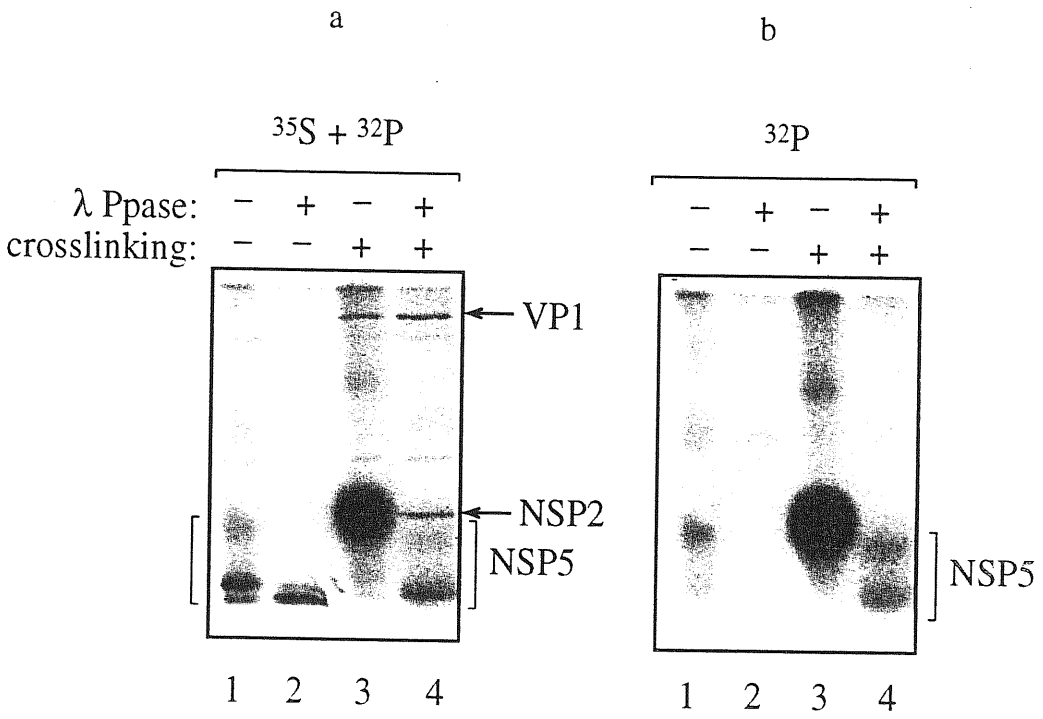


Fig.18. *In vitro* phosphorylation assay of anti-NSP5 immunoprecipitates of crosslinked extracts. In order to see the activity of the crosslinked immunoprecipitates, MA104 infected cells were labeledd with ^{35}S methionine, crosslinked as indicated, immunoprecipitated with anti-NSP5 and subjected to the *in vitro* phosphorylation with [g-^{32}P]ATP. The two panels show the total radioactivity [^{35}S+^{32}P] in **a** and ^{32}P only in **b**. λ-Ppase treatment is indicated.

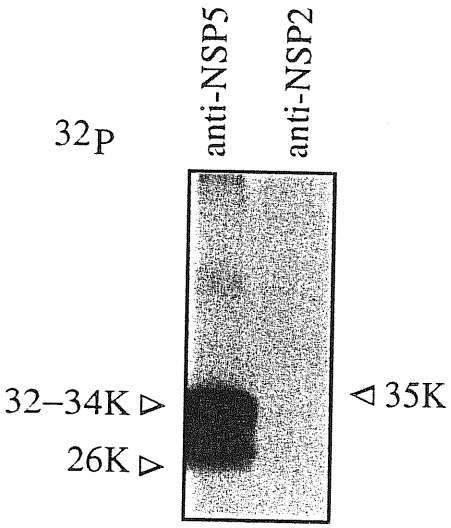


Fig.19. *In vivo* ^{32}P labeling of MA104 rotavirus infected cells.
Lane 1 shows anti-NSP5 immunoprecipitates from extracts of ^{32}P labeled infected cells. Lane 2 represent anti-NSP2 immunoprecipitates from the same extracts. The MWs of NSP2 and NSP5 are indicated.

3.2 Upregulatory role of NSP2 in the NSP5 phosphorylation .

The experiment of *in vitro* phosphorylation in crosslinked extracts suggested that either NSP2 or VP1 or both were responsible for the increased phosphorylation. In order to study the biochemical consequence of NSP5/NSP2 interaction, NSP2 and NSP5 genes were cloned in the pCMV eukaryotic vector under the control of T7 promotor and both constructs cotransfected and expressed in MA104 cells using the vaccinia-T7 (vTF7) expression system. Total extracts from transfected cells were then prepared and analysed by ECL-western blotting assay. As a control MA104 cells were cotransfected with NSP5 and pCMV vector alone.

Figure 20 is a representation of an anti-NSP5 immunoblott of extracts obtained after NSP5/pCMV and NSP5/NSP2 cotransfections. As it can be seen, the presence of NSP2 has a strong effect on NSP5 phosphorylation reflected in the appearance of the hyperhosphorylated 32K band (lane 2). The control (lane 1) demonstrates that NSP5 is also phosphorylated in the absence of NSP2 producing in this case, mainly a band of 28K. When these results are analysed together it appears that NSP2 plays a relevant role in the post-translational modification of NSP5. Thus, NSP2 not only increases the phosphorylation of NSP5 *in vitro* but it also upregulates the phosphorylation of NSP5 *in vivo,* in the absence of other viral proteins, in particular VP1.

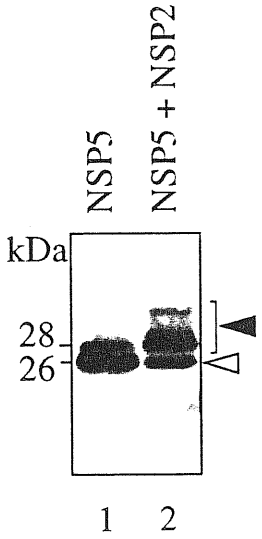


Fig.20. Anti-NSP5 ECL-western blotting of cellular extracts obtained from MA104 cells transfected with NSP5 (lane 1) or cotransfected with NSP5 and NSP2 (lane 2) and expressed by vTF7 system. Filled arrowheads indicate NSP5 phosphorylated forms and open arrowheads the 26K precursor.

4. Biochemical characterization of deletion mutants of NSP5.

To better study the phosphorylation properties of NSP5 and to map the relevant regions of the protein involved in this process deletion mutants of NSP5 were generated. These mutants were also used to investigate the NSP5/NSP2 interaction(s).

4.1 Description of the NSP5 deletion mutants.

The gene 11 which encodes the rotavirus NSP5 protein was truncated from its C-terminal and N-terminal parts to generate NSP5-deletion mutants DC18, ΔC29, ΔC48, ΔC67 and ΔN33, ΔN80, respectively, as it was described in the chapter "Methods" and shown in Fig. 22.

Figure 21 shows the complete aa-sequence of osu NSP5 composed of 197 aa. In Figure 22 the full aa-sequence of the protein was schematically divided in five regions. The first one is 33 aa long very rich in serines and threonines cotaining 35% of the total number of these residues in NSP5. The second and the third regions are less rich in serines and threonines in comparison with the 33 aa N-terminal region. The fourth region is composed of three subregions. Two of them are basic (B) and between them is situated one acidic (A) region of 20 aa. All three subdomains are very rich of charged residues arg, lys, asp, his and glu (60%). The fifth C-terminal region has been indicated as a putative "kinase domain" because of slight homology at its C-terminal 18 aa with the family of guanido kinases (419). Analysis of the full NSP5 sequence by Pile Up and Bestfit of GCG package did not show significant homology of NSP5 with tipical motives of other protein kinases. Probably it may play other roles in NSP5 phosphorylation. This region is

also 100% conserved in all group A rotaviruses and 38% identical between group A and C.

Several deletion mutants of NSP5 were created. Some containing deletion from the N-terminal part like ΔN33 and ΔN80 that lack the first 33 and 80 aa, respectively. Others contain deletions from the C-terminal part of 18, 29, 48 and 67 aa. Of these the ΔC18 lacks only the higly conserved 18 aa motif that follows the highly charged basic-acidic-basic (BAB) domain, while ΔC29, ΔC48 and ΔC67 represent sequential deletions of the basic, acidic and basic regions of this domain, respectively. One double deletion mutant ΔN33/ΔC67 was also constructed. This protein contains only the central core of 97 aa, that could represent an ideal substrate of phosphorylation.

ROTAVIRUS-NSP5

MSLSIDVTSL PSISSSIFKN ESSSTTSTLS

GKSIGRSEQV ISPDAEAFNK VMLSKSPEDI

GPSDSASNDP LTSFSIRSNA VKTNADAGVS

MDSSTQSRPS SNVGCDQVDF SLTKGINVSA

NLDSCVSIST DNKKEKSKKD KSRKHYPRIE

ADSDSEDYVL DDSDSDDGKC KNCKYKKKYF

ALRMRMKQVA MQLIEDL

Fig.21. NSP5 amino acid sequence.

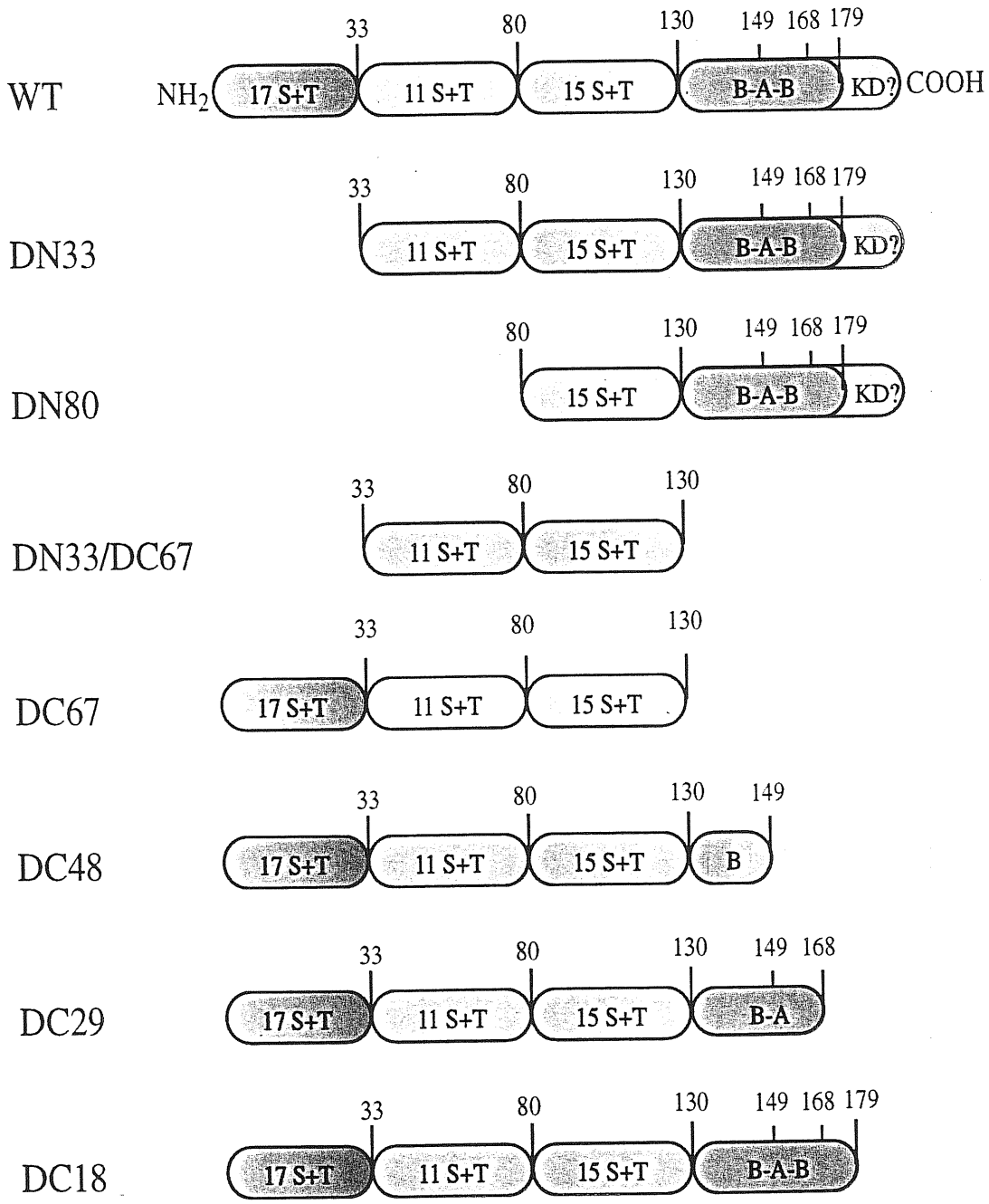


Fig.22. Schematic representation of NSP5 wt and deletion mutants. The content of serines and threonines in each bar are indicated.

4.2 Expression of NSP5 deletion mutants.

All the deletion mutants were cloned in the pCMV expression vector that contains the T7 promotor to generate pCMV/ΔN33, pCMV/ΔN80, pCMV/ΔN33-ΔC67, pCMV/ΔC67, pCMV/ΔC48, pCMV/ΔC29 and pCMV/ΔC18. The complete NSP5 gene in the pCMV/NSP5 construct was used as a control during the transfection experiments.

In order to investigate the behaviour of each mutant *in vivo,* MA104 cells were transiently transfected with each one of the generated constructs. In all cases transfections were followed by vTF7 infection and analysis were performed by western blotting assays.

Figure 23 shows anti-NSP5 immunoblotting analysis of electrophoresed cellular extracts obtained from NSP5-wild type and mutants ΔC18, ΔN33 and ΔC67. As one can see from the figure, NSP5 wt consists mainly of a protein of 26K and a second band of 28K. As expected, by comparison with the C7-MA104 stable transfectant cell, λ-Ppase treatment was able to transform all the products into a single 26K band (Fig 24, lane 2). This results show that, as in the case of C7-MA104, NSP5 alone is phosphorylated *in vivo* although with a pattern different from the one obtained in infected cells. When mutants ΔC18, ΔN33, ΔC67 were expressed, only ΔC67 failed to get any phosphorylation (Fig 23, lane 4) while both ΔC18 and ΔN33 got phosphorylated, albeit, with different efficiency. Interestingly, ΔN33 had a pattern of phosphorylation which resembled more the one observed with the wild type NSP5 in infected cells, being in relative terms more efficient than NSP5 (Fig 24, lane 3). On the other hand, ΔC18 was slightly phosphorylated although not completely negative. This result is important since it shows that the C-terminal 18 aa domain can not be considered by itself the kinase domain.

In all cases λ-PPase treatment confirmed that the high molecular weight bands of the deletion mutants represented the phosphorylated products. When the same analysis

was performed on ΔC29, ΔC48 and ΔN80 no evident phosphorylation was obtained with the exception of ΔN80 where scarce phosphorylation was detected (Fig 26, a, lane3).

4.3 Role of NSP2 on the phosphorylation properties of the deletion mutants.

To investigate the role of NSP2 on the phosphorylation properties of the NSP5 mutants *in vivo,* the NSP2 gene was also cloned in the pCMV vector. The pCMV/NSP2 construct together with each of the mutants constructs was used in cotransfection experiments in MA104 cells and analysed by anti-NSP5 immunoblotting.

As shown in Figure 25 and 26 there was no detectable change in the phosphorylation of any of the previously observed phosphorylated deletion mutants such as ΔN33, ΔN80 and ΔC18. There was no phosphorylation either in the mutants ΔC67, ΔC48 and ΔC29 (Fig. 25 and 26), thus indicating that it was not the lack of NSP2 which affected their ability to be phosphorylated *in vivo* .

The ΔN33 mutant is particulary interesting in this context, since it shows an enhanced phosphorylation in relation to the NSP5-wt, while it is unaffected by the presence of NSP2, suggesting that the N33 domain could participate in the interaction between NSP5 and NSP2.

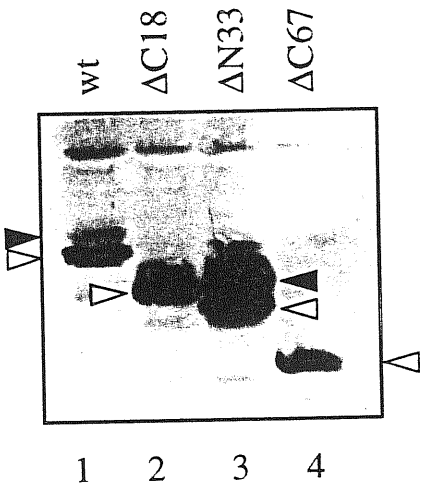


Fig.23. Anti-NSP5 ECL-western blotting of cellular extracts obtained from MA104 cells vTF7-infected and transfected with pCMV/NSP5 (lane 1), pCMV/ΔC18 (lane 2), pCMV/ΔN33 (lane 3), pCMV/ΔC67 (lane 4). Filled arrowheads indicate NSP5 and mutants phosphorylated forms and open one the precursors.

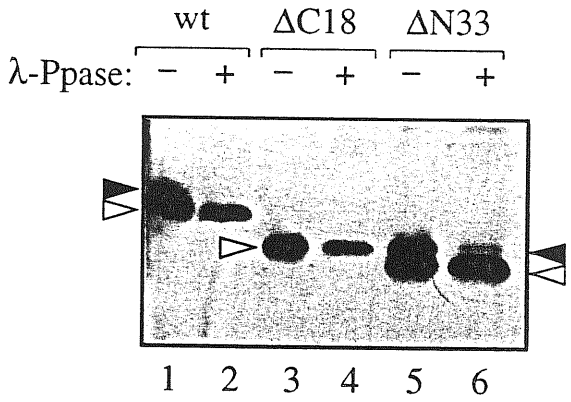


Fig.24. Anti-NSP5 ECL-western blotting of cellular extracts obtained from MA104 cells vTF7-infected and transfected with pCMV/NSP5 (lane 1 and 2), pCMV/ΔC18 (lane 3 and 4), pCMV/ΔN33 (lane 5 and 6). λ-Ppase treatments are indicated.

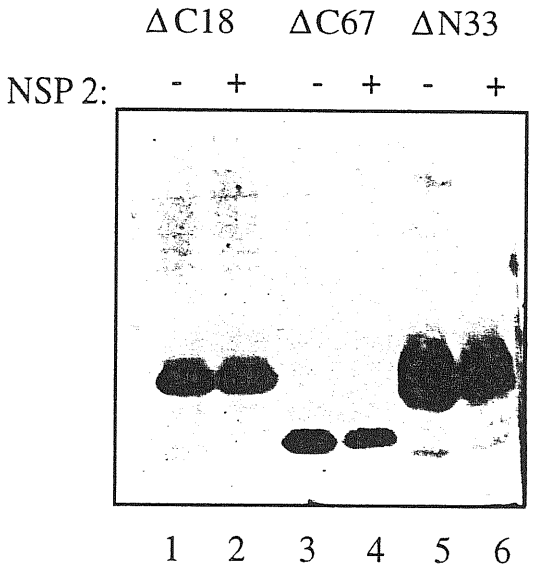


Fig.25. Anti-NSP5 ECL-western blotting of cellular extracts obtained from MA104 cells vTF7-infected and cotransfected with NSP2 and mutants ΔC18, ΔC67, ΔN33 as indicated.

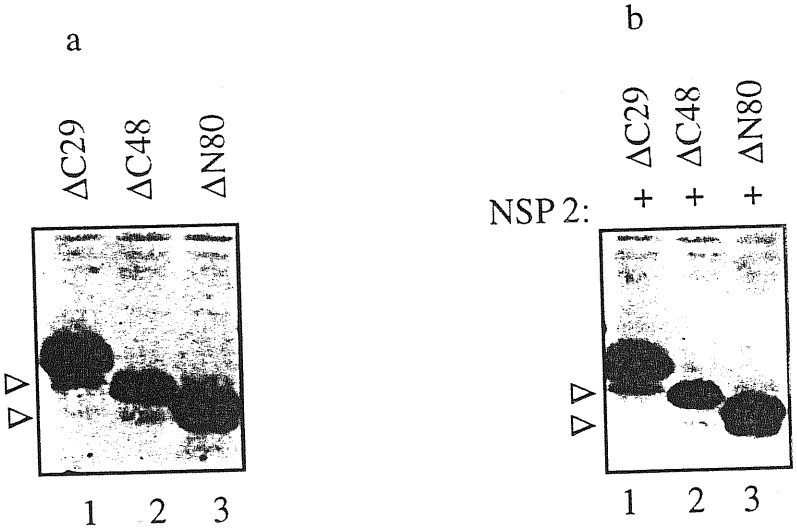


Fig.26. Anti-NSP5 ECL-western blotting of cellular extracts obtained from MA104 cells vTF7-infected and transfected with NSP2 and mutants ΔC29, ΔC48, ΔN80 as indicated in panel **b** or without NSP2 panel **a.** Open arrowheads indicate peptides produced by initiation from internal methionines.

4.4 Co-transfection of NSP5 mutants.

The observation that the removal of the 33 aa N-terminal domain of NSP5 generated a deletion mutant which showed a hyperphosphorylation similar to that of the wt-NSP5 in infected cells, suggested that this domain had some important autoregulatory role on NSP5 phosphorylation. In addition, the demonstration that deletions of the C-terminal region that affected the BAB domain, like ΔC67 and ΔC48 were unable to get phosphorylated also suggested that the phosphorylation activity of the NSP5 protein resides in its C-terminal portion.

Our first approach to discriminate between the substrate portion of NSP5 and its phosphorylation activity was to use the mutants in cotransfection experiments. We decided to use the ΔN33 mutant, which contains an intact C-terminal portion with the ΔC67 mutant as substrate because of its inability to get phosphorylated when expressed on its own. The ΔC67 mutant has intact the N-terminal part, rich of serines and threonines. The ΔC18 deletion mutant was also used as sourse of phosphorylation activity, with ΔC67 as substrate.

As shown in Figure 27 there was no phosphorylation of ΔC67 deletion mutant when co-transfected with ΔC18 (lane1) while a smear was observed over the main ΔC67 band in the ΔN33/ΔC67 cotransfections (lane 2). This result was repeated several times, always showing that ΔC67 can be a substrate in this *in vivo* assay. These results indicated that exist inter-molecular interactions between ΔN33 and ΔC67, which allows the phosphorylation of ΔC67. This mutant seems to be a suitable substrate for studying NSP5 phosphorylation while ΔN33 is very promising as a source of enzymatic activity.

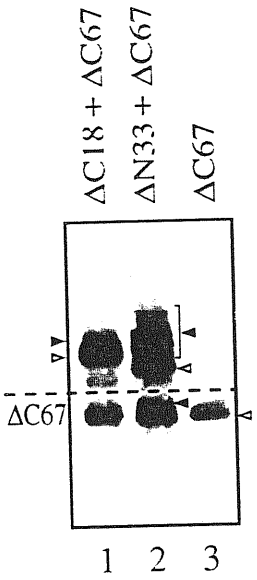


Fig.27. Anti-NSP5 ECL-western blotting of cellular extracts from MA104 cells vTF7-infected and cotransfected with ΔC18/ΔC67, ΔN33/ΔC67 or ΔC67 as indicated. The dotted line indicates the region of the gel below which ΔC67 forms can be seen. Open arrowheads indicate precursors while filled arrowheads indicate phosphorylated forms.

CHAPTER 4

Discussion

NSP5 is one of the least produced rotavirus proteins in infected cells which is localized in the virosomes where viral replication occurs. NSP5 is a nonstructural protein which had been previously described to be phosphorylated (186) and O-linked glycosylated (187).

We have now shown that in virus-infected cells, as well as in the transfectant cell line C7-MA104 the rotavirus NSP5 undergoes a complex process of post-translational phosphorylation. In addition to the two previously identified 26K and 28K NSP5 forms, we have found a new form of 32-34K, which is heterogeneous, judging from the smear observed in the autoradiographies. We also demonstrated that NSP5 can be phosphorylated *in vitro*, with incorporation of phosphates mainly in the 28K and 32-34K bands. Previous reports on NSP5 phosphorylation had indicated that the incorporation of phosphate did not modify the mobility of the protein in (SDS)-PAGE (186). We showed here that this is not the case, as two different phosphatases were able to convert NSP5 in the form of 26K.

As we mentioned at the beginning it had been previously demonstrated that NSP5 was also post-translationally modified by the addition O-linked monomeric residues of N-acetylglucosamine and that β-elimination of [^{35}S]methionine-labeled NSP5 produced a complete conversion of the 28K protein into a smaller 25-26K form (187). The removal of phosphate groups from NSP5 could have also contributed to this change of apparent molecular mass, as they are sensitive to alkaline hydrolysis (415, 416). We now confirmed that, as previously reported, the 26K band was labeled when cells were pulsed with [^{3}H]glucosamine, thus indicating that this small form was modified by O-

glycosylation. The results of λ-Ppase treatment of [^{3}H]-labeled NSP5 demonstrated that all forms can be converted into the 26K form with no loss of carbohydrate moieties.

In brief, our data are consistent with a model in which the addition of phosphates rather than O-glycosylation is the cause of the different mobilities of the forms of 26, 28 and 32-34K. The fact that the 26K form can itself be labeled with phosphate indicates that phosphorylation of particular sites do not produce a change in NSP5 mobility as described also in other proteins (417). Cytoplasmic O-glycosylation appears to modify only slightly the PAGE mobility of NSP5, as revealed by the difference between the *in vitro* translated 25K precursor and the de-phosphorylated 26K immunoprecipitated from virus-infected cells. The 25K *in vitro* translated precursor could be converted into a 28K form when incubated with extracts of infected cells, but no conversion of the 25K form was observed when extracts of non-infected cells were used. That the 28K form is produced by phosphorylation of *in vitro* translated 25K precursor was demonstrated by λ-PPase treatment of the immunoprecipitates.

All this results suggest that NSP5 is not only a phosphoprotein but it probably has autophosphorylation activity. A number of evidences point in this direction: a) there was phosphorylation in the extensively washed immunoprecipitates of NSP5, b) only cellular extracts from infected cells containing NSP5 (and not from MA104 non-infected cells), as well as NSP5 in the form of immunoprecipitate, were able to convert the *in vitro* translated 25K precursor into a 28K form, c) NSP5 was post-translationally modified in the C7-MA cell line (ruling out the need of other viral proteins), while extracts of non-infected MA104 cells were unable to phosphorylate the *in vitro* translated 25K precursor. In addition, treatments of the immunoprecipitates with detergents, high salt and different pH were ineffective in separating the kinase activity from NSP5, and immunoprecipitates of NSP5 were able to phosphorylate, although not very efficiently the exogenous added α-casein substrate (data not shown).

After our results on hyperphosphorylation, other authors expressed NSP5 in E. coli (418, 419). When the E. coli-expressed NSP5 was used after immunoprecipitation in the *in vitro* kinase assay, a signal at the molecular weight 26K was obtained(418, 419). This result again suggests autophosphorylation activity of NSP5 without the presence of any other viral or cellular protein. Apparently, the post-translational O-glycosylation of NSP5 is not essential for protein phosphorylation activity, at least for its modification into 26K form. However it might still be important for full enzyme activity or for interaction with other protein substrates or enzyme regulators.

We, as well as the authors cited above, were not able to obtain 28K and 32-34K multiple phosphorylated forms of NSP5 in *in vitro* re-phosphorylation experiments after λ-PPase de-phosphorylation (data not shown) or from purified NSP5 produced in E. coli, respectively. In the *in vitro* translation assay in the presence of extracts of infected cells we were successful in obtaining a form of 28K, but again it was not possible to generete the 32-34K form. These results supported the idea that NSP5 could be a component of a complex where it has a regulated phosphorylation activity.

One of the possible hypothesis is that cellular protein kinases are involved at some stage of the phosphorylation of NSP5 and that they specifically modulate its phosphorylation activity. In the amino acid sequence of NSP5 from rotavirus SA11 and other strains, there are potential protein kinase C phosphorylation sites (S-X-R/K-R/K-X-X-S and K/R-X-S) at serine residues 22, 30, 75, 100, and 136. There are also serine residues at positions 56, 154, and 165 that are potential sustrates of casein kinase II (S-X-X-D/E) (418). Protein kinase C and casein kinase II have been shown to be active with the P phosphoprotein of vesicular stomatitis virus (420) and various paramixoviruses (421, 422, 423).

We do not exclude that, *in vivo,* CKII or other kinases could modulate NSP5 phosphorylation activity through phosphorylation of specific sites of the protein (421).

Indeed, we were able to obtain NSP5 phosphorylation *in vitro* when immunoprecipitates of NSP5 were incubated with CKII a (one of the two subunits of CKII), obtained from *Xenopus laevis* (139) and kindly supplied by Dr. Germaine Jacob. But we did not detected any difference in the phosphorylation pattern of NSP5 when in a competition *in vitro* kinase assay we used two different substrates for NSP5 phosphorylation [γ^{32}P]ATP and cold GTP (GTP is a better substrate for CK II) (data not shown). In addition CKII a was unable to re-phosphorylate the de-phosphorylated NSP5 immunoprecipitates (λ-Ppase treated) as well as the 25K immunoprecipitated *in vitro* translated product. So, if CKII has any role on NSP5 multiple phosphorylation it should be somehow only part of a complex chain of phosphorylation/de-phosphorylation events which is difficult to be reproduced *in vitro.*

We have also studied by *in vivo* crosslinking assays the interactions of NSP5 with other proteins and their role on NSP5 phosphorylation activity. Interestingly, our results demonstrated close interactions of NSP5 with NSP2 and the viral polymerase VP1. NSP2 is another rotaviral nonstructural protein which has been previously found to interact with the viral polymerase and also to form homomultimers (424). We have now shown that, NSP2 interacts also with NSP5 as demonstrated by DSP and UV-crosslinking experiments.

In the anti-NSP2 followed by anti-NSP5 sequential immunoprecipitations (Fig. 16) we detected different amounts of NSP5 to interact with NSP2 and VP1, suggesting that exist several crosslinked interactions: between VP1 and NSP5 as well as between VP1, NSP2, and NSP5 where NSP5 interacts mainly with VP1 and vice versa VP1 interacts preferentially with NSP5 and less with NSP2. Probably we also have in the anti-NSP2 immunoprecipitates VP1-NSP2 complexes which lacks NSP5 as previously described (424).

Another aspect concerning our *in vivo* crosslinking studies we would like to discuss here is the relation between the type of crosslinker used and the respective interpretation of the results. We have different results regarding the presence of VP1 in the crosslinked complexes obtained by DSP-and UV-crosslinking. By UV-crosslinking we obtained NSP2/NSP5 complexes while VP1 was not detected. DSP chemical crosslinking occurs between proteins at a distances of no more than 12Å (424). By UV irradiation it can be crosslinked only activated groups at a distance of less than 12Å one to the other (425). Since UV crosslinking is usually used to see interactions of proteins with nucleic acids, it could be suggested that we are dealing here with a more complex protein/protein interaction. The possibility that the band of 35K was NSP5 crosslinked with an RNA was also very unlikely since we did not obtain any labeled RNA when we used the 5'-end labeling protocol previously used with NSP2 and NSP3 (data not shown). By the same protocol it was shown (426) that viral ssRNAs bind to NSP2. However, it was also shown that *in vivo* NSP2 can be crosslinked to the 11 dsRNA segments of rotavirus (426). Furthermore these autors described that oligonucleotides bound to NSP2 after RNase treatment migrated in denaturing gels as a series of smeared bands. This heterogenity according to them could result from the fact that various residual amino acids were crosslinked to the oligonucleotide after proteinase K treatment and suggests that several crosslinking sites can be present on this protein. Another possible explanation of this heterogenity could be due to several crosslinked RNA species leading to anomalous migration. These results indicated that various RNAs interact with this protein. On this basis a possible explanation is that the interaction between NSP5 and NSP2 is reinforced when the latter is bound to RNA. By crosslinking NSP2 on RNA by UV irradiation, the NSP2/NSP5 complex could withstand the conditions of immunoprecipitation that otherwise dissociate it. It should be emphasized that the immunoprecipitation conditions

that we used are quite harsh, since washings are made in the presence of SDS/NP-40/DOC.

By two different approaches NSP5 was identified in close interaction with NSP2. We also showed in the *in vitro* phosphorylation assay that NSP5 is much more efficiently hyperphosphorylated in the presence of NSP2 than when NSP2 is missing. When NSP2 and NSP5 were cotransfected we obtained a very similar NSP5-phosphorylation pattern to that one observed in infected cells. It appears that NSP2 upregulates the phosphorylation activity of NSP5. As in hepatitis C virus the degree of phosphorylation of the phosphoprotein P is regulated by another viral protein and this phosphorylation may play an important role in modulating the proliferation of virus-infected cells (87).

A possible role of NSP2 in the cotransfected cells could be that of blocking cellular phosphatases that have some de-phosphorylation activity on NSP5. Another possibility is that NSP2 might be involved as well in the activation of a cellular kinase which would contribute to the hyperphosphorylation activity of NSP5 by phosphorylating specific sites of the protein thus provoking structural change(s) of NSP5 that could favour its further hyperphosphotylation.

Alternatively, NSP2 could by itself interact with NSP5 directly and this might stimulate the NSP5 hyperphosphorylation activity. We do not exclude any of the previous hypothesis but our results support more the idea of direct NSP2/NSP5 interaction, because: a) NSP2 was crosslinked in close interaction to NSP5 by chemical and UV-crosslinking; b) NSP2 contributed to the increase in the hyperphosphorylation of 32-34K band in the *in vitro* phosphorylation experiments as well as in the cotransfection experiments.

The results obtained with the ΔN33 deletion mutant also supports the idea that NSP2 interacts directly with NSP5. Deletion of the N-terminal portion of NSP5

contributed to an enormous increase in the phosphorylation of the ΔN33 single transfected mutant in respect to the wild type transfected NSP5 (Fig. 20). This result suggests that the hyperphosphorylation of NSP5 is a regulated process. The fact that NSP5 also was hyperphosphorylated in infected cells let us to support the idea that NSP2 interacts preferentially with the N-terminal portion of NSP5 and this interaction produces as a consequence, a structural modification of NSP5 which contributes to increse its hyperphosphorylation. In other words, the N-terminal portion of NSP5 plays the role of an inhibitory domain of the NSP5 hyperphosphorylation. For instance, the N-terminal regions of P phosphroteins from negative-sense RNA viruses have been shown to be critical for both protein structure and function (427). It has been also identified two important amino terminus domains of the Sendai virus P protein: one plays a role in genome replication and the other participates in a stable interaction with the free nucleoprotein NP (428).

The capacity of the ΔN33 deletion mutant to phosphorylate ΔC67 also implies the existence of a particular intermolecular interaction of NSP5 in which are engaged different regions of the protein.

In our laboratory we have also performed anti-NSP5 and anti-NSP2 immunocytochemical analysis of cotransfected MA104 cells with NSP2 and the wild type NSP5 or the deletion mutants (data not shown). By these assays we found that cotransfected NSP2 and NSP5 form a virosome like structures (VLS). This preliminary results are the first demonstration that two rotavirus nonstructural proteins togheter are responsible for the formation of VLS. Previously, after cotransfections of different virus structural proteins virus-like structures were obtained, which are different from the VLS. For example VP4, VP6 and VP7 from Bovine rotavirus strain C486 were cloned and expressed in a baculovirus expression system. Combination of these proteins were assembled into a series of virus-like particles (429). Other authors demonstrated that

simultaneous expression of VP2 and VP6 in mammalian cells resulted in the formation of intracellular spherical particles resembling double-layered rotavirus particles (430). Recently it was reported that coexpression of the nucleoprotein (N) and the phosphoprotein (P) of human respiratory syncytial virus as well as of N and P from rabies virus was sufficient to induce the formation of N-P complexes or for the generation of cytoplasmic inclusions (431, 432). However, no VLS were observed when NSP2 was cotransfected with any of the deletion mutants and no change in the deletion mutants phosphorylation properties was observed when they were cotransfected with NSP2. These results strongly suggest that perhapes NSP2 does not interact only with the N-terminal deleted region of NSP5 but probably interacts with both the amino-terminal and carboxy-terminal portion of the protein. For example in the phosphoprotein P of rabies virus at least two independent binding sites for the N protein were identified ; one is located in the carboxy-terminal part of the protein and another between amono acids 69 and 177 in its amino-terminal part. The formation of cytoplasmic inclusions seems to require the presence of both N-binding sites on the P protein (431). We have produced now internal deletion mutants of NSP5. If cotransfection of these mutants with NSP2 is followed by VLS formation it would strongly support our idea that at least two independent NSP2-binding sites exist on NSP5.

In summary we have demonstrated that: a) NSP2 binds NSP5; b) NSP2 upregulates the hyperphosphorylation of NSP5; c) NSP2 is not essential for the initial step of the NSP5-phosphorylation; d) NSP2 does not influence the phosphorylation pattern of any of the deletion mutants; e) NSP2 does not participiate in VLS formation with any of the amino-and carboxy-terminal deletion mutants; f) only cotransfection of the wt-NSP5 with NSP2 is sufficient for VLS formation.

Another interesting point related with the formation of NSP2/NSP5 complexes is the observation that the viral polymerase VP1 also participates in them. We have

demonstrated for the first time that the rotavirus polymerase interacts with NSP5. Our results also showed that VP1 interacts mainly with NSP5 and less with NSP2 as shown by the sequential immunoprecipitation experiment. What is the biological significance of this interaction at present remains a matter of speculation. We think that since the three identified proteins (NSP2, NSP5 and VP1) are found in the virosomes of infected cells during the replication step they could participate in this process. Particulary, the role of NSP5 and NSP2 could be structural, in the formation of the replicative complex and probably regulatory related with the initiation of viral replication. Recently, using various monoclonal antibodies and specific antisera against the structural (VP1, VP2, and VP6) and nonstructural (NSP1, NSP2, NSP3, and NSP5) proteins, a viral RNA-protein complex with replicase activity was recovered using a monoclonal antibody directed against NSP2. This complex contained the structural proteins VP1, VP2, and VP6 and the NSP2 and was able to complete nascent negative strands but did not use exogenous RNAs as template (426). Numerous phosphoproteins have been implicated in the replication of RNA viruses as well (433, 434, 435, 436). Our finding that NSP5 consists of a wide range of isoforms created by differential phosphorylation, carefully upregulated by NSP2, introduces a level of heterogenity that could be used to distinguish the different viral mRNAs. The diversity of NSP5 forms might make it possible to control the packaging of the right set of genes in the viral particles.

On the basis of the results we have and the data available until now I would like to propose a model, although very speculative, just to simply ilustrate a possible mechanism of action of the complex described above. It is possible that NSP2 captures from the cellular pool viral ss (+)strand RNA (NSP2 has demonstrated ssRNA binding activity). NSP2 could then function to bring the 11 different RNAs to the virosomes and interact with NSP5 which might discriminate segments depending on the degree of phosphorylation. Once the RNAs are recognized VP1would starts to replicate them

Despite the set of results we have in support to NSP5-autophosphorylation activity or NSP5 kinase activity we can not be certain to say that NSP5 is a kinase. Further work is needed in this direction. Protein kinases contain a common catalytic domain which typically is found in a 250-to 300 amino acids polypeptide segment, including the binding site for ATP and the protein substrate (258). NSP5 in this respect is smaller. Therefore, potential regulation of its protein kinase activity is likely to be achieved by complex formation with other polypeptide(s) that provide the regulatory functions. Analysis of the NSP5 amino acids sequence, using the computer algorithm described by Bairoch et al. (330), showed no significant homology with other protein kinases. Hence, NSP5 appears to be exceptional in the large group of protein kinases that have the typical amino acid motives (418). Slight homology with the family of guanido kinases in the last 18 aa at the carboxy-terminal end of NSP5 and putative nucleoside triphosphate binding site at the amino-terminus of the protein has been described (418, 119). However, we have been unable to find any homology analysing NSP5 sequence by PILE UP and BESTFIT programmes from GCG package. Our results obtained with NSP5 deletion mutants showed that deletion of the carboxy-terminal end with the basic-acidic-basic domain completely remove NSP5 phosphorylation activity. Deletion only of the last C-terminal 18 amino acids did not completely removed NSP5 phosphorylation activity but drastically influence the interaction of the protein with NSP2. On the other hand the removal of the amino-terminal 33 amino acids stimulated NSP5-phosphorylation activity to hyperphosphorylation. These results made us to believe that the NSP5 kinase domain could reside in the C-terminal half of the molecule while the N-terminal portion could act as a regulatory domain for NSP5 hyperphosphorylation. Further analysis of NSP5 mutants could help to precisely define the regions required for the protein phosphorylation activity.

Summary

NSP5 (non-structural protein 5) is one of the two proteins encoded by genome segment 11 of group A rotaviruses. In virus infected cells NSP5 accumulates in the virosomes and is found as two polypeptides of molecular masses of 26 and 28K proteins. NSP5 has been previously shown to be post-translationally modified by the addition of O-linked monosaccharide residues of N-acetylglucosamine (O-GlcNAc) and also by phosphorylation. We have now found that, as a consequence of phosphorylation, a complex modification process gives rise to previously unidentified forms of NSP5, with molecular masses of up to 34K. Treatment with phosphatases of NSP5 obtained from virus-infected cells produced a single band of 26K. NSP5 could be phosphorylated *in vitro* by incubation of immunoprecipitates with [γ-^{32}P]ATP, producing mainly phosphorylated products of 28 and 32-34K. In both, *in vivo* and *in vitro* phosphorylated NSP5, phosphates were only found incorporated in serine and threonine residues. The *in vitro* translated NSP5 precursor polypeptide of 25K could also be phosphorylated and transformed into a 28K protein, by incubation with extracts obtained from virus infected cells, but not from non-infected cells. In addition, NSP5 labeled *in vivo* with [1,6-^{3}H]glucosamine showed only the presence of the 26K and 28K proteins (converted to 26K by λ-Ppase treatment) suggesting that the type of protein produced is regulated according to the level of phosphorylation and/or O-glycosylation.

We have also demonstrated by *in vivo* crosslinking experiments that NSP5 binds NSP2, another rotavirus nonstructural protein, and the viral polymerase VP1. Our results also suggest that NSP5 interacts mainly with VP1 and less with NSP2. In addition we have observed that NSP2 upregulates NSP5 phosphorylation in *in vitro* phosphorylation assays as well as *in vivo* in cotransfections experiments without the presence of VP1. However, NSP2 is not required for NSP5 initial phosphorylation as we

observed in NSP5 transfection experiments that the protein was phosphorylated up to the 28K form in the absence of NSP2.

NSP5, NSP2 and VP1 have been previously found in the virosomes of infected cells where viral replication occurs. It was also demonstrated that NSP2 forms multimers and has RNA-binding activity. Recently it has been reporteded that VP1 interacts with NSP2. Now we demonstrate that NSP5 is also part of VP1-NSP2 complexes. On the basis of these results and our findings an speculative model could be proposed in which NSP5 hyperphosphorylation could have a role in packaging of ss(+)RNA during replication.

The expression of NSP5 in transfectant cell line, without the presence of any other viral protein, is characterized by the 26 and 28K forms, but no 32-34K form was observed, which is characteristic for the NSP5 from infected cells. To better study NSP5 phosphorylation we have produced N-terminal and C-terminal deletion mutants. Interestingly, ΔN33, a deletion mutant lacking the 33 aa N-terminal domain, has a phosphorylation pattern similar to the NSP5 produced in infected cells. This result suggests an autoinhibitory role for the 33 aa N-terminal domain on NSP5 wt phosphorylation. On the other hand, the fact that the phosphorylation of NSP5, when cotransfected with NSP2 resembles ΔN33 suggests that somehow NSP2 interacts with the N-terminal part of NSP5. However we did not obtain any effect of NSP2 on the phosphorylation of the ΔN33 or any mutants. This means that: a) their phosphorylation is independent of the presence of NSP2; and b) NSP2 probably interacts with more than one part of NSP5. Further experiments are needed to characterize in details the phosphorylation properties of NSP5 in respect to its interaction with NSP2.

The results that we have until now also suggest that NSP5 is not only a phosphoprotein but that also has autophosphorylation activity: a) there was phosphorylation in the immunoprecipitates of NSP5, b) only cellular extracts from

infected cells containing NSP5 (and not from MA104 non-infected cells), as well as NSP5 in the form of immunoprecipitate, were able to convert the *in vitro* translated 25K precursor into a 28K form, c) NSP5 was post-translationally modified in the C7-MA cell line (ruling out the need of other viral proteins), while extracts of non-infected MA104 cells were unable to phosphorylate the *in vitro* translated 25K precursor.

Analysis of NSP5 amino acid sequence, by PROCITE database as well as by PILE UP and BESTFIT programmes of GCG package revealed no significant homology with other protein kinases. Hence, NSP5 appears to be exceptional, since it does not contain any of the typical amino acids motifs found in the large group of protein kinases.

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