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Studies on rotavirus NSP5 phosphorylation and its interaction with NSP2

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To my family

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I have thought about what to write in these acknowledgements and after one hour of staying here (in front to the computer), I have decided to write these acknowledgements in Spanish because is the language that I prefer to express my gratitude.

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SUMMARY

Rotavirus NSP5 is a non-structural protein that localises in cytoplasmic viroplasms of infected cells. NSP5 interacts with NSP2 and undergoes a complex post-translational hyper-phosphorylation, generating species with reduced PAGE mobility. This process has been suggested to be due in part to autophosphorylation. Here, we show that it rather works as an autoregulator of its own phosphorylation though the activation of cellular kinases. In this thesis, it is described the development of an *in vitro* phosphorylation assay using as a substrate an *in vitro*-translated NSP5 deletion mutant that was phosphorylated by extracts from MA104 cells transfected with NSP5 mutants but not by extracts from mock-transfected cells. The phosphorylated products obtained showed shifts in mobility similar to what occurs *in vivo*. From these and other experiments, we concluded that NSP5 activates a cellular kinase(s) for its own phosphorylation. Three NSP5 regions were found to be essential for kinase(s) activation. Glutathione–S-transferase-NSP5 mutants were produced in *E. coli* and used to determine phosphoacceptor sites. These were mapped to four serines (153, 155, 163 and 165) within an acidic region with homology to casein kinase 2 (CK2) phosphorylation sites. CK2 was able to phosphorylate NSP5 *in vitro*. NSP5 and its mutants fused to enhanced green fluorescent protein were used in transfection experiments followed by virus infection and allowed the determination of the domains essential for viroplasm localisation in the context of the virus infection. A second hyper-phosphorylation assay was also developed. This is an *in vivo* assay in which, two constructs are co-transfected. One of them tagged with 11 aa served as substrate while the other was used to map the domains required to induce activation of the cellular kinase. We learn that the two activities can be separated, demonstrating that the hyper-phosphorylation is a process *in trans*, with one molecule activating and the other being phosphorylated. We described a motif *a* (from amino acids 63 to 67) in region 2 with the amino acidic sequence SDSAS. This motif, and in particular phosphorylated serine 67, is responsible to trigger the hyper-phosphorylation process. In fact, mutation of serine 67 to

aspartic acid in the full-length NSP5 allowed hyper-phosphorylation of NSP5 in the absence of NSP2, suggesting that NSP2 could produce a conformational change in NSP5 to expose motif *a* (serine 67), thus allowing phosphorylation of serine 67. On the other hand, NSP5 substrate characteristics were mapped in region 4 (amino acids 131 to 179). The serines 153, 155, 163 and 165, that are CK2-like phosphorylation sites, are in part responsible for the hyper-phosphorylation. Two other serines, 137 and 142, that are a putative sites for PKC phosphorylation are good candidates to be also substrates in NSP5 hyper-phosphorylation. Moreover, the c-terminal tail (T) of NSP5 of 18 aa was found to be also necessary for activation of the cellular kinase. Although its role is not yet clear, it is possible that a dimersation *in trans* with another NSP5 molecule can explain the results. Alternatively, a direct interaction with a cellular kinase may be required to permit its activation

Viroplasms are discrete structures formed in the cytoplasm of cells sustaining rotavirus replication that constitute the machinery of replication of the virus. In this thesis, it has been investigated the relative localisation of NSP5 and NSP2 within viroplasms as well as the dynamics of viroplasm formation in cells infected with rotavirus that also express NSP5 or NSP5 fused to EGFP. The results showed NSP2 localising more internally with respect to NSP5. The number of viroplasms was shown to first increase and then to decrease in post-infection time, while the area of each one increased, suggesting a fusion between them. The interaction between NSP2 and NSP5 was investigated using two different assays, namely a two-hybrid system and an *in vivo* binding assay. Both methods gave essentially the same results, indicating that in NSP5 the N-terminal region (33 aa) as well as C-terminal part (amino acids 131 to 198) are required for binding to NSP2. These two regions were able to confer to EGFP ability to localise in viroplasm and to form VLS with NSP5.

CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS LIST

aa	amino acids
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ci	Curie
CK2	casein kinase 2
C-terminal	carboxy-terminal
DLP	double layered particles
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DSP	dithiobis[succinimidylpropionate]
dsRNA	double strand RNA
DTT	dithioeritrol
EDTA	ethylenediamine tetraacetic acid
EGFP	eukariotic green fluorescent protein
FCS	foetal calf serum
GST	glutathione-S-transferase
GTP	guanosine triphosphate
h	hour
HCV	hepatitis C virus
HIV-1	human immunodefiency virus-1
HRP	horse radish peroxidase
HSV-1	herpes simplex virus-1
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo Dalton
λ-Ppase	lambda protein phosphatase
min	minute
MOI	multiplicity of infection
MW	molecular weight
NSP	nonstructural protein
N-terminal	amino-terminal
ORF	open reading frame
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffer saline
PFU	plaque forming units
PKC	protein kinase C
RSV	respiratory sincytial virus.
RVV	rift valley virus
SDS	sodium dodecyl sulfate
VP	viral structural protein

INTRODUCTION

History

In 1973 Bishop (24) and collaborators discovered a 70 nm human rotavirus and its association with severe endemic diarrhoea in infants and young children. This agent identified on direct visualization by electron microscopy, using thin–section electron microscopy of duodenal mucosa. Shortly afterward, rotavirus was identified in faeces by electron microscopy by Flewett *et al.* (85), Bishop *et al.* (23), and others (136).

It soon became apparent that the 70 nm particle, was an important etiologic agent of diarrhoea of infants and young children, causing about 35-50% of the hospitalisations for this form of gastroenteritis during the first 2 years of life (134). In a relatively short period, investigators from many countries reported the detection of rotaviruses in faeces of paediatric patients with diarrhoeal illness, and it was soon established that rotaviruses were the long–sought-after major viral etiologic agents of severe diarrhoea of infants and young children in both developed and developing countries, consistently outraking in importance other know etiologic agents of severe diarrhoea.

Although the human rotaviruses were discovered in 1973, it should be noted that in 1963, by Adams and Kraft, using thin section EM, described virus–like particles in intestinal tissue of mice infected with epizootic diarrhoea of infant mice virus (2). These particles were similar to those observed by Bishop et al.,(24). In 1963, Malherbe and Harwin (166) described the isolation of a 70 nm virus designated SA11 (simian agent 11). This virus, derived from rectal swab obtained from healthy vervet monkey, was recovered in vervet monkey kidney cell culture. In 1967, Malherbe and Strickland-Cholmley (167) described another virus similar to SA11, the O (offal) agent, which was isolated in vervet monkey kidney cell culture from mixed washings of intestines of cattle and sheep. In 1969, Mebus et al (184) reported successful cultivation of the Nebraska calf diarrhoea virus (NCDV) in primary foetal bovine cultures; in 1972, Fernelius *et al.*(81) reported that the NCVD resembled the reoviruses morphologically but

was distinct antigenically. The murine, simian, O and bovine agents were later found to exhibit characteristic rotavirus morphology and to share a group antigen with other rotaviruses (86, 135).

Virus classification

The rotaviruses compose a genus within the family *Reoviridae*, and rotaviruses share common morphologic and biochemical properties. Salient features are that (a) mature virus particles are about 100 nm in a diameter and posses a triple–layered icosahedral protein capsid composed of an outer layer, intermediate layer and an inner core layer; (b) 60 spikes extend from the smooth surface of the outer shell; (c) particles contain an RNA-dependent RNA polymerase and others proteins capable to produce 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; and (h) the viruses exhibit a unique morphogenic pathway (i.e., virus particles are formed by budding into ER) and enveloped particles are evident transiently at this stage of morphogenesis. Mature particles are non-enveloped, and these virions are liberated from infected cells by cell lysis or by no classic vesicular transport in polarized epithelial cells (35, 72).

Rotaviruses are classified serologically for the presence of multiple groups (serogroups) and for the existence of multiple serotypes within each group. A rotavirus group (or serogroup) includes viruses that share cross-reacting antigens detectable by a number of serological tests, such as immunofluorescence, ELISA and immunoelectron microscopy. Rotaviruses comprise seven distinct groups (A to G). Group A, B, and C rotaviruses are those currently found in both humans and animals whereas viruses groups D, E, F, and G have been found only in animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses

in different groups (299). The group antigenic determinants are found on most of the structural proteins and probably on many of the non-structural proteins as well. This has been documented by showing that monospecific antisera and some monoclonal antibodies specific for individual polypeptides cross-react with strains in addition to those to which they were made. However, cross-reactive epitopes on the inner capsid protein (VP6) are those usually detected by ELISA diagnostic.

Group A rotaviruses have been established as causing significant diarrhoeal disease in the young. Group B rotaviruses have been associated with annual epidemics of severe diarrhoea primarily in adults in China (264). Group C viruses have been sporadically reported in faecal specimens from children with diarrhoea and several outbreaks; their clinical significance remains unclear. Rapid diagnostics tests as ELISA and monoclonal antibodies to detect non-group A rotaviruses have been established, and these are beginning to facilitate determining the clinical importance of these viruses (201, 300). Few non-groups A rotaviruses strains (a single C porcine, group C human virus, and group B porcine virus) have been cultivated (246, 250, 258, 292). This inability to grow most non-group A rotaviruses has hampered obtaining information on these viruses, although gene-coding assignments and sequence data have been obtained (35, 72). Classification into serotypes is based on the antigenic differences in the proteins of the outer capsid, VP7 and VP4. The first, a glycoprotein, determines the G-type specificity, and the second, the P-type specificity, owing to its protease sensitivity. At present, there are 15 G types (237), with G1, G2, G3 and G4 being the predominant ones throughout the world (52). However, there have been reports of infections by unusual G types (1, 19, 61, 103, 251, 262), and recently there have been reports of the emergence of serotype G9 in several countries, such as Brazil (251), Malawi (61), the USA (106, 232), Argentina (28), the UK (122) and Australia (208, 295).

The genetic system

A Reassorting genetic system

The segmented nature of the genome suggests that, like other segmented genome viruses (reovirus (234) and influenza virus (207)), rotaviruses undergo recombination by a mechanism of reassortment. A corollary of recombination by a mechanism of reassortment is that recombination frequencies are expected to be high or undectably low, with no continuum between low and high frequencies (82, 275). Physical evidence for ressortment (177) and a high frequency of recombination (236) have both been reported for rotaviruses. The 11 genome segments of the parental virus strains can theorically reassort into 2^{11} different possible genome constellations, if reassortment is random. No study has identified all possible reassortant progeny from a cross, presumably because insufficient progeny have been examined. Selection for fitness seems likely to limit the viable constellations. However, reassortment of genome segments among viable progeny generally appears to be random during infection of cultured cells (233) and animals (97). Reassortment may be somewhat restricted in certain cases that involve parental viruses that are distantly related (287). Reassortament can also be restricted by the imposition of selective pressure during the growth of progeny virus, such as selection against temperature-sensitive (*ts*) mutants present in one or both parents (98, 99).

Random reassortment of genome segments in rotaviruses is expected to occur at high frequency, because two alternative parental cognates for each segment are avaible to be packaged. In studies where progeny were analysed without selection, reassortants dominated over wild-type parentals among the progeny and segment segregation appeared to be relatively random (98). However, in cases where two (*ts*) mutants were crossed and the frequency of the wild-type reassortant progeny was determined, recombination was either high or undetectably low (236). In the case in which temperature was used to select ts^+ reassortants, the frequency of ts^+ progeny was significantly lower, being in the range of 1-20% of the progeny.

Reassortment and vaccination

Reassortment has been used to generate vaccine candidate strains for use in a modified "Jenerian" approach to protection of children from rotavirus disease (133). In this approach, revelant neutralizing antigen-encoding genome segments for human rotavirus are moved by reassortment onto a recipient genetic background that consists of an animal virus of know avirulence and immunogenecity in humans. In the reassortant, the antigen of a virulent virus is presented to the host in the context of the remainder of the genes derived from an avirulent virus, leading to the development of a protective immune response without a disease response to the vaccine virus. Human neutralization antigen—encoding genes have been moved by reasssortment onto recipient backgrounds of bovine strains UK or WC3 and rhesus strain RRV for the production of vaccine candidate strains (235)(see below).

Clinical aspects and vaccines

Rotavirus gastroenteritis continues to cause substantial morbidity and mortality worlwide, despite widespread breastfeeding and use oral rehydration therapy. In developing countries, it has been estimated that more than 600000 to 870000 children deaths from rotavirus infection every year (94, 95, 139). The vast majority of which are in developing countries (39, 62, 279). Among the etiological agents of acute infectious diarrhoea rotaviruses account for nearly 25% of hospital admissions in India with vomiting and diarrhoea followed by severe dehydration in very young children below 2 years of age. In the United States, rotavirus is a common cause of hospitalisations, emergency room visits, and out-patient clinic visits. It has been estimated that one in every 73 children will have been hospitalised because of diarrhea due to rotavirus A during the first 5 years of life (96). Several European studies point to rotavirus as the agent responsible for 20-60% of cases of gastroenteritis requiring hospitalisation (40, 127, 176, 196, 245, 266). In Australia, similar figures have been reported (16). As far as "non-group A" rotavirus is concerned, group B rotavirus has been identified in

epidemic outbreaks of severe diarrhea in adults in Southeast Asia since 1982 (80), and in symptomatic infections in children. Outbreaks of diarrhea due to group C rotavirus have been identified in Asia (206), Brazil (260), and Europe (44, 284), and outbreaks of sporadic gastroenteritis caused by this virus in children have been observed in the USA (126), Japan (146), and the UK, with frequencies ranging from 1% to 6.8% (124, 295).

The GAVI (Global Alliance on Vaccines and Immunizations) Task Force on Research and Development identified rotavirus vaccine as 1 of the 3 priority vaccines for the international development. This burden of disease indicates that effective, safe rotavirus vaccine is needed, and in 1998 the first rhesus-human reassortant rotavirus tetravalent vaccine, Rotashield, was licensed in the United States. This vaccine is a live, oral vaccine incorporating four strains of rotavirus, a rhesus rotavirus strain with human serotype G3 specificity and three single-gene human-rhesus reassortants for human serotypes G1, G2 and G4. The VP7 protein found in the outer capsid of the virus determines G serotypes. Fourteen G serotypes have been identified, but only five are important in humans. Serotype G1 is the most common, followed by serotype G3, and serotypes will vary from year to year in any geographic location. In RRV-TV the parent rhesus strain by single–gene reassortment so that each contains 10 genes from the parent rhesus strain along with a single gene encoding the VP7 protein from human rotavirus strain G1, G2 or G4. Each dose of vaccine contains 10^5 PFU of each component rotavirus strain (65). However, the recommendations for its use were withdrawn in 1999 because of the recognition of an uncommon but serious adverse event, intussusception. This pathogenesis remains an enigma. An experimental murine model for intussusception induced by lypopolisaccaride, Murine rotavirus strains enhanced the rate of intussusception in this model, causing an enlargement of the mesenteric lymph nodes and Peyer's patches. However, Peyer's Patches did not appear to be the anatomic lead point for intussusception. The investigators further demonstrated differences in the ability of rotavirus strains to increase the murine incidence of intussusception. Some strains of rotavirus could be a cofactor in the induction of acute intussusception in mice. They were not able to

demonstrate obstructive intussusception. Additional evidence of the variation in responses in mice to different strains of rotavirus, where virus was detected in mesenteric lymph nodes and Peyer's patches after inoculation of simian-human reassortants. The implications of these findings for humans, however, are unclear. Three possible hypotheses could be the cause of the intussusception. One is the " unique strain hypothesis", according to which the induction of the intussusception is related to the rhesus rotavirus vaccine strain but not to other vaccine or wild-type strains. The second is that of the 'bolus dose", in which ingestion of an inoculum of high viral titer by an infant is necessary to induce this disorder. A third is the "viral replication" hypothesis in which wild-type rotaviruses are a rare but consistent cause of the disease.

Of note, China is the only country that has a rotavirus vaccine in use, a lamb-derived strain. Very limited data are available about its efficacy and safety, including no information on the occurrence of intussusception in association with its administration (215).

Rotavirus proteins

The mature virion of group A rotavirus is formed by three concentric layers of protein that enclose a genome composed by 11 segments of dsRNA. The inner most layer of the virion is formed by 60 dimers of protein VP2, which surrounds the viral genome and 12 copies each of RNA polymerase VP1 and guanylyltransferase VP3. The intermediate layer is formed by 260 trimers of VP6 and the outermost layer is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is made up of 780 copies of glycoprotein VP7, organized as trimers, while 60 spike-like structures, formed by dimmers of VP4, extend approximately 12 nm from VP7 surface (10).

Core and inner capsid proteins

VP1

The structural protein VP1, is codified by the gene 1 of rotavirus (173). This protein is the most highly conserved among the rotaviruses of groups A (PO-13

(avian rotavirus), SA11, Gottfried, RF), B (ADRV, IDIR) and C (Cowden) (34, 55, 69, 89, 121). For the bovine rotavirus (RF strain) has been determined that the segment contains a single ORF that extends 1088 codons and possesses 5'- and 3'-terminal untranslated region of 18 and 20 bp, respectively. The AUG conforms with to the Kozak consensus sequence and yields a protein with an apparent molecular weight of 125 kDa (55). In the beginning was demonstrated that the inner shell proteins of the virus constituted by VP1, VP2 and VP6 and the purified the subviral particles conformed by core proteins VP1 and VP2, but contain significantly less VP6 protein, has the transcriptase activity (114). This function was adjudicated by Valenzuela *et al.*, 1991, using 8-azido ATP an analogue of nucleotide (280). Posterior demonstration using SA11 mutant, *ts*C, which carries a mutation in the gene coding for the viral RNA polymerase, shown that affects both transcription and minus-strand synthesis (197). Also, it was demonstrated that VP1 can interact with NSP2, forming complexes with VP1 and viral messenger RNA, that can coordinate RNA packaging and assembly of the viral cores (137) and with NSP5 when is chemically cross-linked in living cells (4). VLPs (virus-like particles) formed by VP1/VP2 constitute the minimal replicase particle in the *in vitro* replication system (304). The fact that VP1 recognizes the 3' end of the viral mRNA is not sufficient for the replicase activity. Because it was demonstrated that the absence of other viral proteins, VP1 lacks replicase activity. The presence of VP2 stimulates VP1 replicase activity several fold. This suggests that VP2 must interact with the RNA-protein complex before VP1 gains replicase activity (210, 213). The affinity for RNA in the case of VP1 is sequence specific (211).

VP2

VP2 is codified for the genomic segment 2 (34, 79, 173), yielding a myristylated protein of around 94 kDa (54). In the different group of rotavirus, this protein is antigenic distinct between them (78). This protein form part of viroplasms (216) and part of the inner shell of the virus (90, 173), corresponding to the most abundant protein of the inner shell. VP2 can bind unspecifically dsRNA in cells

infected with rotavirus (31). Also, it was seen that in replicative subviral particles isolated from SA11 strain, VP2 is associated to the RNA polymerase VP1 (114). Other evidence, using *ts*F (a termosensitive mutant of the SA11 strain that encoding for VP2, where is present a substitution to an Ala→Asp at position 387), demonstrated that subviral particles recovered from cells infected with this virus have a 20 fold less replicase activity. This indicates that VP2, but not VP6, is an essential component of enzimatically active replicase particles (169, 170). It was seen that VP2 expressed in baculovirus is able to bind DNA and RNA and to form core like particles of 45 nm in diameter. Also, heterologous expression in baculovirus of VP2 and VP6 are able to form single shelled particles, demonstrating the existence of an innermost protein shell in rotavirus, which is formed independently of other rotavirus protein. Same results were demonstrated with an expression vaccinia system for these two proteins (101, 148, 303). Posterior studies, indicates that three-dimensional structure of the VP2 layer are formed of 120 molecules, with each dimmer extending neighbouring fivefold axes, where the amino termini of VP2 molecules are located near the icosahedral vertices (47, 152). The ability of VP6 to interact with VP2 was examined. Indicating that the hydrophobic interactions between VP6 to VP2 residues are responsible for the stability of DLP (double-layered particles). Also the subtle electrostatic interactions between VP6 and the underlying transcriptase machinery can be essential for mRNA synthesis (46)

The binding domain to RNA of VP2 was localized between amino acids 1 to 132 (147), part of the N-terminal corresponding to aminoacids 1 to 25 are important for the binding with VP1 (302). Suggesting that this protein must interact with the RNA-protein complex formed between VP1 and viral mRNA, before VP1 gains a replicase activity (210). The binding in the replication of VP2 corresponds to bind the mRNA template for the minus–strand synthesis (213). Recently, it was described with a baculovius system, that VP2 could interact with the non-structural protein NSP5 (21).

VP3

VP3 is codified for the gene 3 of rotavirus group A, corresponding to a protein of 88 kDa located in the viral core particles (156, 192). The first description of this protein indicated that its forming precores replication intermediate in ensemble with VP1, VP2, NSP5, NSP2 and NSP3 (90). VP3 was identified, in purified viral particles, as a guanyltransferase due to the binding to GTP, resulting in the formation of a GpppG cap (89, 220). Expression of VP3 in a baculovirus system showed that the expressed VP3 covalently bound GTP. These suggest that VP3 alone is a guanyltransferase (155). For the encapsidation of VP1 and VP3 in the inner core is necessary an interaction with the N-terminal of VP2 (302). Evidences shown, that VP3 has affinity for ssRNA but not for dsRNA. While the ssRNA-binding activity of VP3 was found to be sequence independent, the protein does exhibit preferential affinity for uncapped over capped RNA (213) (211). Also, a methyl transferase activity was detected to VP3 of open cores, detected by binding to S-adenosyl-l-methionine (SAM). This is a substrate necessary for cap methylation of RNA. Combined results suggest that the guanyltransferase and methyltransferase are both components of VP3 and, therefore, that VP3 is a multifunctional capping enzyme (48).

VP6

VP6 is the major structural component of virions, and it plays a key role in virion structure because of its interactions with both outer capsid proteins VP4 and VP7 and the core protein VP2. VP6 spontaneously form trimers (74) and is extremely stable. These trimers can be dissociated and reassembled by changing pH (278). Removal of VP6 from double-layered particles results in a loss of polymerase activity, but it is unknown whether VP6 plays a structural or functional role in this process (22, 249). Analyses of deletion mutants, virus variants, *ts* mutants, and chimeric proteins have begun to dissect the domains of VP6.Thus, the trimerization domain is reported to be between amino acids 246 and 315, and a domain necessary for the formation of double layered particles is located between amino acids 281 and 397 (3, 278). Proline 308 has been implicated in trimer stability. The N-terminus is predicted to be an amphipatic α-helix and to be

crucial to virus assembly, possibly by functioning in transporting VP6 to viroplasmic inclusions (170). Studies with MAbs to VP6 have suggested that amino acids 58 to 65 are not accessible in the viral particles, and these could be located at the interface with VP2; other domains may be accessible trough the outer shell of the virus (277).

Outer capsid proteins

VP4

VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration. The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity. After attachment of the virus to the cell surface, it must penetrate the plasma membrane to productively infect the cell. This penetration is increased by, and most probably is dependent on, trypsin treatment of the virus, which results in the specific cleavage of VP4 to polypeptides VP8 and VP5 (157); cleavage of VP4 does not affect cell binding and is rather associated with entry of the virus into the cell. The mechanism of activation of the virus infectivity by trypsin is not know, although it is believed that penetration of virus may be promoted by terminal regions of VP4 newly generated by trypsin cleavage or by a possible conformational change in the VP4 molecule resulting from this cleavage (10). A number of functional domains have been described on VP4: i) a trypsin-sensitive region; *in vitro* treatment of virions with trypsin results in specific cleavage of VP4 (776 amino acids) at arginines 231,241 and 248, and yields polypeptides VP8 (aminoacids 1-231) and VP5 (242-776), with concomitant enhancement of viral infectivity (12, 157); ii) most rotavirus strains are able to agglutinate red blood cells, and this agglutination has been shown to be mediated by the interaction of VP4 with sialic acid (SA) on the surface of erytrocytes. It has been shown that the domain responsible for this interaction is located between aminoacids 93 and 208 in the VP8 cleavage fragment of VP4 (87), with amino acid residues 155 and 188-190 playing an essential role in the SA-binding activity of the protein (120); iii) the integrin-binding motif DGE present in the VP4 at the amino acids 308-310 has been reported as used by variant nar3 to bind to the α2 I domain of integrin

α2β1 (105, 301) ; iv) in RRV VP5 there is a single disulfide bridge between Cys-318 ans Cys-380; it was shown that Cys-267, present in nar3 but not in wt RRV, is able to form an alternate disulfide bond with Cys-318 (60). And preliminary results indicate that infectivity of these two viruses is differentially affected by treatment with reducing agents; v) prediction of the secondary structure of VP4 suggests that it has at least two different structural domains. One comprising the amino terminal 60% of the protein, is predicted to be a globular domain rich β-strands, while the second domain, represented by the remaining carboxy-terminal part of VP4, is rich in long stretches of α-helix (73, 158). One stretch of 63 residues has a predicted coiled-coil structure and vi) the VP5 fragment contains a hydrophobic region between amino acids 384 and 404 that shares some sequence similarity with an internal fusogenic hydrophobic domain of the e1 glycoproteins of Sindbis and Semliki Forest viruses (165), although functionality of this region in rotaviruses has not been established (10).

VP7

VP7 is an outer capsid glycoprotein that is the second most abundant protein species in the virion. Biochemical analyses rapidly determined that VP7 is a glycoprotein that contains only N-linked high mannose oligosaccharides, which are processed by trimming (11, 70, 130). $Man_8GlcNAc_2$ and $Man_6GlcNAc_2$ oligosaccharide residues are found on intracellular VP7 and $Man_6GlcNAc_2$ (and to a lesser extent $Man_5Glc\text{-}Nac_2$) is found on mature virus particles (130, 131). VP7 is cotranslationally glycosylated as it is inserted into the membrane of the ER, and insertion is directed by a cleavage signal sequence found at the amino-terminus of the protein (29, 71, 130). The ORF of 326 amino acids begins with an initiation codon with a weak consensus sequence. A second, in-frame initiation codons precedes two regions of hydrophobic aminoacids (H1 and H2), which can act as the signal sequence to direct VP7 to the ER, although the second is thought to be the major species used in cells. A third in-frame initiation codon is also present downstream from the second hydrophobic domain in some strains.

The site of cleavage of the signal peptide in VP7 is glutamine 51, and this amino-terminal residue in VP7 from purified virus is blocked by pyroglutamic acid (263). Studies to determine the signals specifiying retention of VP7 in the ER have shown that VP7 does not contain the sequence KDEL found to confer retention for some other ER proteins (198). Two regions (one spanning amino acids 51 to 61, and the second between amino acids 61 and 111) that mediate retention of VP7 in the ER have been identified (225, 228, 263, 294). Further sequence analyses identified a consensus peptide LPXTG [STGAE], where X indicates any other amino acid, within the ER retention domain of VP7 (180). This sequence is of interest because it is found in bacterial surface proteins and is proposed as being responsible for a posttranslational modification necessary for proper anchoring of proteins to the bacterial plasma membrane. Although these residues are critical for retention, the method by which VP7 remains in the ER is unresolved. After its insertion into membranes, VP7 is resistant to digestion with proteolytic enzymes, suggesting it is not a membrane-spanning protein (70, 130). Structural reconstructions of virion VP7 have shown it is trimeric and VP7 form an oligomers with other proteins (VP4 and NSP4) in infected cells (163). These oligomers, and protein rearregements and interactions with calcium, appear to be important in the assembly of VP7 into the outer capsid (67, 226, 227, 255).

Nonstructural proteins

NSP1

The non-structural protein 1 is codified by the gene 5, is a minor protein of 486 to 495 amino acids. This is an RNA-binding protein that contains a cysteine–rich region and is a component of early replication intermediates. Near the amino terminus, NSP1 contains three basic regions and a cysteine-rich domain, suggesting that this area is responsible for the RNA-binding activity of the protein. The amino acidic motif present in the cysteine-rich region is C-X2-C-X8-C-X3-H-X-C-X2-C-X2-C-X5-C, forming one or two zinc fingers (117). Analysis with deletion mutant of NSP1 demonstrated that the RNA-binding domain resides within the first 81 amino acids of the protein and that the highly conserved

cysteine-rich region within this region of the protein is essential for the activity. Analysis of intracellular fractions of cells infected revealed that large amounts of NSP1 were present in the cytosol and in association with the cytosketeletal matrix (116). For another hand, NSP1 can interact with other viral proteins NSP3, NSP5 and NSP6 (100). Analysis of interaction with cellular proteins, shown an interaction with interferon regulatory factor 3 (IRF-3). The result suggests that a binding domain resides in the C-terminus of NSP1 and that the N-terminal conserved zinc finger is important but not sufficient to mediate binding to IRF-3. The role of this interaction in rotavirus-infected cells could be inhibit the activation of IRF-3 and diminish the cellular interferon response (104).

NSP2

The segment 8 of rotavirus group A codifies the non-structural protein 2. This protein was found in viroplasms of infected cells (217). In addition, NSP2 in rotavirus infected-cells, bind ssRNA and dsRNA of the 11 genomic segments (8, 138). Also, NSP2 can form 10S multimers without the presence of RNA and other viral proteins (137). This RNA-binding of the NSP2 multimers was determined to be non-specific and to have a strong preference for ssRNA over dsRNA. Enzymatic analysis revealed that NSP2 possessed an associated nucleoside triphosphatase (NTPase) activity *in vitro*, which presence of Mg^{2+} catalysed the hydrolysis of each of the four NTPs to NDPs with equal efficiency (267). Posterior studies demonstrated that the NSP2 multimers correspond to an octamer that is functional in the binding of RNA and ADP. The presence of Mg^{2+} can produce a partial dissociation into smaller oligomers (253). In addition, a helix-destabilizing activity was associated to this protein (269). The X-ray structure of the functional octamers of NSP2 was resolved with a definition of 2.6 Å. Presenting the NSP2 monomer has two distinct domains. The N-terminal domain has a new fold and the C-terminal domain, that resembles the ubiquitous cellular histidine triad (HIT) group of nucleotydil hydrolases. This structural similarity suggests that the nucleotide-binding site is located inside the cleft between two domains. A prominent grooves that run diagonally across the

doughnut-shaped octamer are probable locations for RNA binding. Several RNA binding sites, resulting from quaternary organization of NSP2 monomers, may be required for the helix destabilizing activity of NSP2 and its function during genome replication and packaging. Analysis with the temperature sensitive for NSP2 (*tsE*) at non-permissive temperature, confirm that NSP2 is required form virus replication. (125, 270).

Other evidences, suggest that NSP2 is involved in the replicative cycle is that form part of a viral complex (90, 212) in presence of structural proteins VP1, VP2 and VP6 (9, 137) and interact directly with NSP5 in viroplasms. Moreover, NSP5 *in vivo* phosphorylation was enhanced by co-expression with NSP2 (4). These two proteins are able to form viroplasms-like structure (VLS) in absence of other viral proteins and rotavirus replication (77). Also, functional and structural homologies were described among NSP2, bluetongue protein NS2 and reovirus σNS, suggests that they are functional homologs (268, 269).

NSP3

NSP3 is a non-structural protein codified by the segment 7 of rotavirus group A. This protein is a RNA binding protein. The RNAs linked have been identified as rotavirus mRNAs and the sequence correspond to the 3'end sequence common to all rotavirus group A and C genes (221). The sequence established as the minimal RNA sequence required for binding of NSP3 is GACC (222, 223). By two hybrid system in yeast and co-immmunoprecipitation in infected cells was isolated eukaryotic initiation factor 4GI (eIFG4I). In addition, the amount of poly (A) binding protein (PABP) present in the complexes decreases during rotavirus infection, even if the transcriptional elements eIF4A and eIF4E remain unaffected. These results show that a physical link between the 5' and the 3' ends of mRNA is necessary for the efficient translation of viral mRNAs and strongly support the closed loop model for the initiation of translation. Suggesting that NSP3 take the place of PABP on eIF4GI making responsible for the shut-off of cellular protein synthesis (190, 219). Moreover, the RNA binding domain was mapped between amino acids 4 and 149. NSP3 also forms dimers and one of

them binds one molecule of RNA and that dimersation is necessary for strong RNA binding. The dimersation domain was mapped between amino acids 150 and 206.The eIF4GI binding domain has been mapped in the last 170 amino acids of its C-terminus. Establishing that, NSP3 is composed of two functional domains separated by a dimersation domain (100, 218). Moreover, these two functional domains are required to enhance the translation of viral mRNA, demonstrating that NSP3 is a functional analogue of the cellular PABP (282). Once that, the X-ray structure of the NSP3 RNA binding domain bound to a rotaviral mRNA 3' end has been determined. Showing that NSP3 is a novel, heart-shaped homodimer with a medial RNA binding cleft. This homodimer is asymmetric, and contains two similar N-terminal segments plus two structurally different C-terminal segments that intertwine to create a tunnel enveloping the mRNA 3' end. Biophysical studies demonstrate a high affinity binding leading to increased thermal stability and a slow dissociation kinetics, consistent with the NSP3 functions (66, 202). On the other hand, the X-ray structure of the C-terminal domain of NSP3 recognizing a fragment of eIF4GI was determined. Showing that, the homodimersation of NSP3 yields a symmetric, elongated, largely alpha-helical structure with two hydrophobic eIF4G binding pockets at the dimmer interface. Site-directed mutagenesis and isothermal titration calorimetry documented that NSP3 and PABP use analogous eIF4GI recognition strategies, despite marked differences in tertiary structure (108, 281).

On the other hand, recently data suggest that NSP3 plays a significant role in viral growth in the gut and spread to peripheral sites in mice infected with different strains of the rotavirus group A. This mechanism is under investigation (195).

NSP4

NSP4 is the only non-structural protein that does not bind RNA. NSP4 has been studied extensively because it plays a role in viral morphogenesis and is important in virulence by functioning as an enterotoxin. NSP4 is glycoprotein that has multiple domains. This protein is a 20 kDa primary translation product, it is

cotranslationally glycosylated to 29 kDa species, and oligosaccharide processing yields the mature 28 Kda protein that is a transmembrane protein of the ER (70, 130). The 175-amino acid polypeptide backbone of NSP4 consistes of an uncleaved signal sequence, three hydrophobic domains with two-N-linked high mannose glyocosylation sites being in the first hydrophobic domain, and a predicted amphipathic α-helix that overlaps a folded coiled-coil region. The H2 transmembrane domain traverses the ER bilayer, and the C-terminus, which is hydrophilic, forms an extended cytoplasmic domain (30, 45, 71, 130). The carbohydrate moieties remain sensitive to endoglycosidase H digestion, and oligosaccharide processing of the Man9Glc Nac, with the mannose-9 species predominating (30, 130), indicating that no further trimming occurs in the Golgi. NSP4 plays a distinct role in virus assembly. The C-terminal cytoplasmic domain (amino acid 161 to 175) functions in viral morphogenesis by acting as an intracellular receptor on the ER membrane (14, 261, 271). NSP4 binds newly made double-layered particles into the ER lumen. A receptor role for the NSP4 is reported by the observation that double-layered particles bind to ER membranes containing only NSP4 (13, 189). The amphipathic α-helix region, distinct from the receptor domain, is predicted to adopt an α-helical coiled-coil structure, and this region is thought to mediate oligomerization of the virus-binding domains into a homotetramer. Glycosylation of NSP4 is not required for its binding activity to double-layered particles or for oligomerization, but it is required for interaction with calnexin. Heteroligomers of NSP4, VP4 and VP7 have been detected in enveloped particles and calcium has been shown to be important for oligomerization of these proteins in the ER and for proper folding of VP7 epitopes and outer capsid assembly. Recently, a fraction of VP4 and VP7 has been detected at the cell surface of the virus-infected cells, and a cleavage product of NSP4 has been detected in the media of cells before release of virus.

In 1996, NSP4 was shown to induce age-independent diarrhea in mice that mimics disease caused by rotaviruses. This provides one possible explanation for how NSP4 might function as a virulence factor as determined by reassortant analyses. Further work, showed that NSP4 functions as an enterotoxin and that

the extracellular administration of NSP4 to the intestinal mucosa or to crypt cells from mice, and to human intestinal cell lines, can trigger a signal transduction pathway that leads to mobilization of intracellular calcium [Ca^{2+}] and chloride secretion. The current model for the mechanism of action of intracellular and extracellular NSP4 indicates this protein may reduce glucose absorption in epithelial cells as well as induce chloride secretion. NSP4 can also affect the cytoskeleton in polarized epithelial cells, but how these pleitropic properties of NSP4 influence the function of NSP4 in morphogenesis and pathogenesis remains to be clarified. Immunization with NSP4 can induce immunity that protects neonatal mice from disease induced by virus. The identification of NSP4 as the first viral enterotoxin highlights similarities between viruses and other microbes that cause diarrheal diseases and emphasizes a greater complexity of toxin action and gastrointestinal track-pathogen cross-talk than previously recognized (72).

NSP5

NSP5 is one of the proteins encoded by genome segment 11 (291) of group A rotavirus and is characterized by high (24%) serine and threonine content (102, 291). NSP5 was originally described as a polypeptide of 26 kDa. However, immunoprecipitation experiments from virus-infected cells using specific anti-NSP5 antibodies demonstrated that two main forms are produced with molecular masses of 26 and 28 kDa, of which the latter corresponded to a phosphorylated and O-glycosilated form. The O-glycosilation is a cytoplasmic post-translational addition of O-linked monosaccharide residues of N-acetilglucosamine (O-GlcNAc)(102). This modification is present in many proteins localized to the cytoplasmic and nucleoplasmic compartments of the cell (111). On the other hand, the phosphorylation of NSP5 appears as a complex process giving rise to a number of isoforms with different SDS-PAGE mobilities. In particular, species with relative molecular masses of up to 32-34 kDa represent hyper-phosphorylated forms, that can be detected as a relevant fraction of all the NSP5 protein present in virus-infected cells (figure 1). A similar pattern of bands could

be observed when NSP5 was immunoprecipitated from virus-infected cells labelled *in vivo* with $^{32}P_i$. In this case, the 32-34 kDa band became more evident while the 26 kDa protein was less labelled. Phosphatase treatment demonstrated that the modification were due to addition of phosphate (figure 1). These results indicated that part of the newly synthesised NSP5 in virus-infected cells was in the hyper-phosphorylated form.

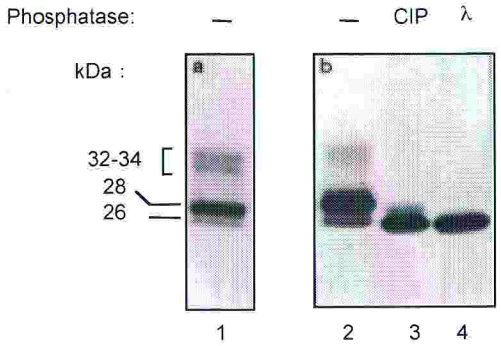


Figure 1. SDS-PAGE analysis of NSP5. a) Immnunoblot analysis of extracts of SA11-infected MA104 cells (4h post-infection) reacted with anti-NSP5 serum. b) Immunoprecipitation of NSP5 from virus-infected cells labelled *in vivo* with [^{35}S]-methionine. From Afrikanova *et al.*,1996 (5)

NSP5 could be also phosphorylated *in vitro* by incubation of the immunoprecipitates with [γ-^{32}P] ATP, producing mainly phosphorylated products of 28 and 32-34 kDa. In addition, the *in vitro* translated NSP5 precursor polypeptide could also be phosphorylated and transformed into a 28 kDa protein by incubation with extracts obtained from virus-infected cells, but not from infected cells (5), thus suggesting that NSP5 phosphorylation may be regulated to some extent by interactions with other viral proteins.

The relation of O-glycosylation and phosphorylation in the various NSP5 isoforms showed that the hyper-phosphorylated forms (32-34 kDa) appear to contain very little or none the O-glycosidic residues. Moreover, partial acid hydrolysis studies revealed only phosphoserine and phosphothreonine in both phosphorylated and hyper-phoshorylated forms (5, 25).

The interaction of NSP5 between NSP2 and the viral polymerase VP1 (4, 224) was demonstrated in experiments of co-immunoprecipitation after *in vivo* chemical (DSP) or UV-induced crosslinking of virus–infected cells (figure 2).

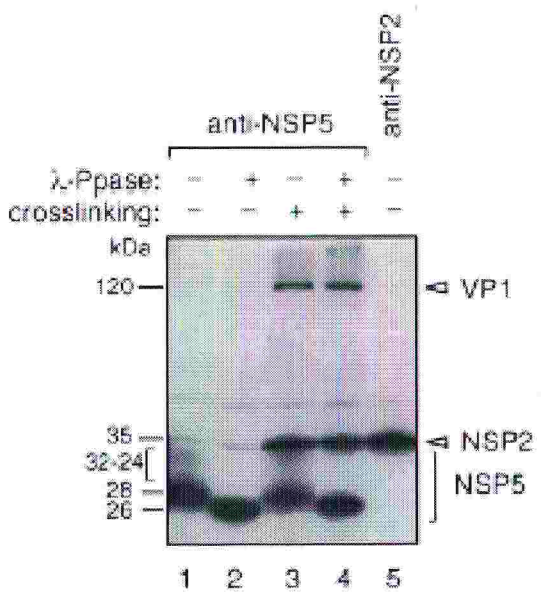


Figure 2. Immunoprecipitation from DSP-crosslinked or non-crosslinked extracts of [^{35}S] methionine labelled MA104 infected cells. Extracts were treated with λ-Ppase before SDS-PAGE analysis, where indicated. As a control, immunoprecipitation with anti-NSp2 is also shown (lane 5) (4).

Interestingly, the NSP2-NSP5 interaction appears to have functional consequences regarding the level of phosphorylation of NSP5. A clear demonstration that NSP2 alone was sufficient for the up-regulation of NSP5 hyper-phosphorylation *in vivo*, was shown in co-expression of NSP5 and NSP2 in absence of other viral protein. The different NSP5 isoforms were visualized by Western immunoblotting. When both NSP2 and NSP5 were present, a clearly increased phosphorylation and hyper-phosphorylation of NSP5 were obtained (figure 3). Demonstrating that a direct interaction between NSP2 and NSP5 is essential for the NSP5 modifications that give a rise to the hyper-phosphorylated forms of 32-34 kDa (4).

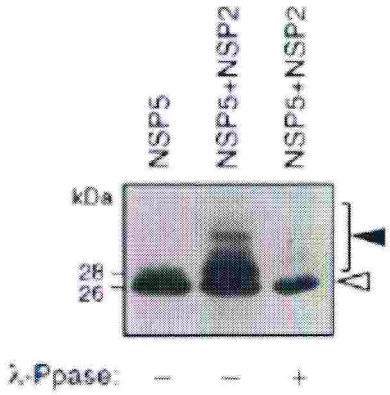


Figure 3. Anti-NSP5 western immunoblot of cellular extracts of MA104 cells transfected with pT7v-NSP5 or co-transfected with pT7v-NSP5 and pT7v-NSP2, as indicated. λ-Ppase treatment of the extract was performed before PAGE. Open and solid arrowheads indicate the NSP5 26 kDa precursor and phosphorylated forms respectively (4).

In other studies, a number of NSP5 deletion mutants were investigated regarding their ability to be phosphorylated *in vivo*. Hyper-phosphorylation of wtNSP5 and mutants was assessed by Western immunoblot because phosphorylation largely affects the migration on SDS-PAGE and also by the degree of phosphorylation by $^{32}P_i$ *in vivo*-labelling and immunoprecipitation. Two mutants with deletion in the first N-terminal 33 amino acids (Δ1) or internal region from amino acids 81-130 (Δ3), showed strong hyper-phosphorylation as evidenced by the reduced mobilities in Western immunoblot and $^{32}P_i$ labelling (Figure 4). The low mobility bands were also the ones with stronger ^{32}P incorporation. None of the other N- and C-terminal deletion mutants (Δ1Δ2, ΔT, ΔC29, Δ48 and Δ4T) showed a change in mobility, even though Δ1Δ2, ΔT and ΔC29 became phosphorylated. Of the two other internal deletion mutants, Δ2 and Δ4 neither was phosphorylated (77).

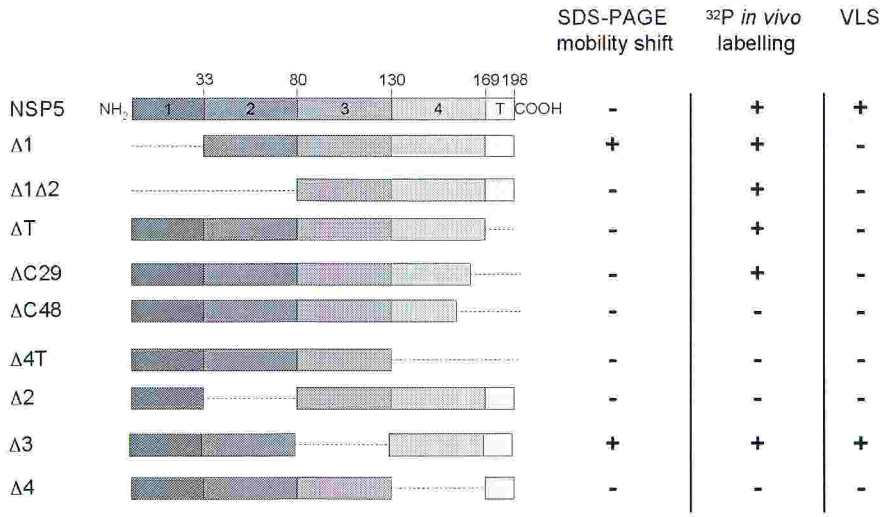


Figure 4. Schematic representation of the NSP5 mutants constructed. The relative amount of Ser/Thr for region 1,2 and 3 is 52,23 and 30%, respectively. The ability of each protein to produce mobility shift, to be phosphorylated *in vivo* and to form VLS is also indicated (77).

In addition, NSP5 was also shown to interact with NSP2 in the absence of any other viral protein of the virus replication. The co-expression of both proteins has a dramatic effect on the localization of either protein, leading to the formation of discrete structures that were called VLS (viroplasms-like structures). Analysis by confocal microscopy demonstrated a precise co-localization of NSP2 and NSP5 figure 5) (77).

The analysis of NSP5 deletion mutants to form VLS in the presence of NSP2 showed that both N- and C-terminal domains of NSP5 are essential. The relation between NSP5 hyper-phosphorylation and its interaction with NSP2 and localisation to viroplasms has not yet been fully investigated (figure 4).

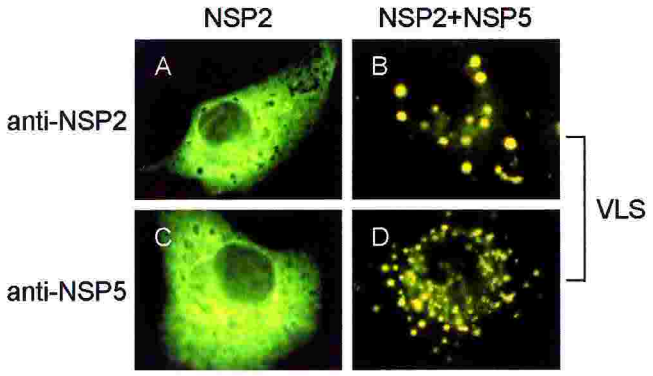


Figure 5. Immunofluorescence of VLS: panel. Localization of rotavirus NSP2 and NSP5. The two proteins were analysed by immunofluorescence microscopy using specific antibodies for NSP2 or NSP5 as indicated, in cells transfected with either NSP2(A) or NSP5 (C) or co-transfected with NSP2 and NSP5 (B,D)(77).

NSP6

Genomic segment 11 of SA11 strain contains a shorter alternative reading frame of 93 amino acids beginning at base 80. This out of phase ORF is conserved with some variations in other rotaviruses, as the human strains Wa, 69M, B7, DS-1 and RV-5, the porcine strains C60 and the bovine strains UK and C7/183. But not in the porcine OSU strain which encodes an ORF of only 51 amino acids (unpublished data). Other viruses as rotavirus group A strains Mc323 and Alabama or group C strains does not encodes NSP6 in the 11 gene. NSP6 corresponds to a protein of 12kDa and is expressed during rotavirus infection. In infected cells, NSP6 was localized in discrete foci, possibly viroplasms, even at late times post-infection (181). It was shown that NSP6 can interact with NSP5 and seems that this interaction is due to the 35 C-terminal amino acids of NSP5, overlapping the multimerization domain of the protein, suggesting that NSP6 might have a regulatory role in the self-association of NSP5 (100, 276).

In Table 1, is presented a summary of the eleven segments of the dsRNA with the proteins that are codified by each segment. The main functional and structural characteristics of each the viral protein is described.

Table 1.: The dsRNA segments and proteins of simian rotavirus A/SA11.

Genome Segment Size [bp]	Gene Product(s) (':Protein Function)	Location in Virus Particle	Copy Number/ Particle	Protein Size aa [Da]	Cognate Proteins‡	GenBank Accession Number(s)	Functions and Properties
1 [3302]	VP1 (Pol)	Inner capsid, 5-fold axis	<25	1088 [125005]	Orthoreovirus lambda3(Pol) Orbivirus VP1(Pol) Coltivirus VP1(Pol) Cypovirus Pol	X168	Fully conservative RNA-dependent RNA polymerase , Part of minimal replication complex (213)
2 [2690]	VP2	Inner capsid	120	880 [102431]	o: VP3 r: l1	X16831	Inner capsid structural protein (22), Sequence non-specific RNA-binding activity (31), Myristoylated (54), Cleaved(76) Part of minimal replication complex (213)
3 [2690]	VP3 (Cap)	Inner capsid, 5-fold axis	<25	835 [98120]	o: VP4 r: l2	X16062 X16387	Guanylyltransferase (155, 220), Methyltransferase (48), Basic Protein (154, 192), Part of virion transcription complex (50)
4 [2362]	VP4	Outer capsid spike	120	776 [86782]	-	D16346 X14204	VP4 dimers (229) form outer capsid spike protein (7, 229), interacts with VP6 (256). Virus infectivity enhanced by trypsin cleavage of VP4 into VP5* and VP8* (157), Hemagglutinin (84, 132), Cell attachment protein (159, 244, 301), P-type neutralization antigen (115), VP5* permeabilizes membranes (64).
	VP5*			529 247-776 [60000]	-		•
	VP8*			247 1-247 [28000]	-		•
5 [1611]	NSP1	Nonstructural	0	495 [58654]	-	L18944 X14914	Associates with cytoskeleton (116). Extensive sequence diversity between strains (144), Two conserved cysteine-rich zinc-finger motifs (193), RNA binding (116).
6 [1356]	VP6 (T13)	Middle capsid	780	397 [4816]	o: VP7	L15384 L33365 M27824	Major virion protein (243), Middle capsid structural protein (231), Homotrimeric 4° structure (243), Subgroup antigen (132), Myristoylated (54), Hydrophobic (170).
7 [1049]	NSP3	Nonstructural	0	315 [34600]	-	M87502	Homodimer (218), Specifically binds 3' end of rotavirus mRNA (221, 223), Binds eIF4G1 (219), Involved in

							translational regulation (282).
8 [1059]	NSP2 (VIP)	Nonstructural	0	317 [36700]	o: NS2 r: sNS	L04531	Non-specific ssRNA-binding (138), Accumulate in viroplasm (217), Involved in viroplasm formation (77), NTPase activity (267), Homomultimer (4-8 subunits) (267), Binds NSP5 and VP1 (4, 138), Regulates NSP5 autophosphorylation (4).
9 [1062]	VP7	Outer capsid glycoprotein	780	326 [37368]	-	K02028	Outer capsid structural glycoprotein (70, 174), G-type neutralization antigen (115), N-linked high mannose glycosylation and trimming (70), RER transmembrane protein, cleaved signal sequence (71).
10 [751]	NSP4	Nonstructural	0	175 [20290]	-	AF087678	Enterotoxin (15), Receptor for budding of double-layer particle through ER membrane (13, 189), RER transmembrane glycoprotein (71), N-linked high mannose glycosylation (70)
11 [667]	NSP5	Nonstructural	0	198 [21725]	-	X07831 M28347	Interacts with NSP2, VP2 and NSP6 (4, 21, 100, 224), Homomultimerizes (100, 224), O-linked glycosylation (102), (Hyper-phosphorylated (5, 291), Autophosphorylation activity (5), Binds ssRNA (181).
	NSP6	Nonstructural	0	92 [11012]	-		Product of second, out-of-frame ORF (181), Interacts with NSP5 (100), Localizes to viroplasm (181).

: Protein structure/function: RNA **pol**ymerase = A (Pol)@; **cap**ping enzyme = A(CaP)@; Inner virus structural protein with **T = 13** symmetry = A(T13)"; **v**iral inclusion body or **v**iroplasm matrix **p**rotein = A(ViP)@. Other species within the genus may have proteins with significant differences in sizes.
† Segments numbered based on migration of SA11 genome segments in SDS-PAGE gel. Migration order may differ among other members of the genus.
‡ Proteins with similar functions from other genera.
* Modified from Prasad web site.

Replicative cycle

Entry of virus

After attachment to the cell surface, the virus must penetrate the plasma membrane to productively infect the cell. This penetration is increased by trypsin treatment of virus, which results in the specific cleavage of VP4 to polypeptides

VP8 and VP5 (157); cleavage of VP4 does not affect cell binding and is rather associated with entry of the virus into the cell. The mechanism of activation of the virus infectivity by trypsin is not known. Although it is believed that penetration of the virus may be promoted by terminal regions of VP4 newly generated by trypsin cleavage or by possible conformational changes in the VP4 molecule resulting from this cleavage. Ultrastructural studies have suggested that rotavirus can enter the cell by both endocytosis and direct cell membrane penetration (72, 73); however, the fact that rotavirus infection is not inhibited by lysomotropic agents that raise endosomal pH or by drugs that affect endocytosis or intracellular transit of endocytic vesicles has been taken as evidence against this mode of entry. Direct cell membrane penetration has thus been alternatively proposed as the mechanism of entry of rotaviruses; nevertheless, evidence supporting this mechanism is rather indirect and mainly suggests that non-endocytic route used.

During the entry process of rotaviruses into the cell, the viral transcriptase is activated and the virus genome transcribed. Transcriptase activity is observed *in vitro* when surface proteins VP4 and VP7 are removed from mature triple-layered virus particles by treatment with calcium chelating agents, a treatment that causes uncoating of the virus and yields double-layered particles (73). It has been suggested that penetration of the virions into the cell's cytoplasm, which has a calcium concentration several orders of magnitude lower than the extracellular media, might be the factor uncoating of the virus and activates the transcriptase (161). However, other evidences suggests that an increase in the intracellular concentration of calcium ions by different mechanisms does not affect onset of viral protein synthesis, suggesting that low intracellular calcium is not essential for initiation of the viral replicative cycle (59). Different rotavirus strains display different requirements to bind, and thus infect, susceptible cells. Cell attachment of some of rotavirus strains isolated from animals is greatly diminished by treatment of cells with neuramidase (NA), indicating the need for sialic acid (SA) on the cell surface. Interaction with a SA-containing receptor, however, does not appear to be essential, because NA-resistant variants that no

longer need SA to infect cells can be isolated (160, 185). In addition, many animal rotavirus strains are naturally NA-resistant, and most if not all strains isolated from humans are also NA-resistant (53, 186). Gangliosides GM1 and GM3 as well as 300-330 kDa glycoproteins have been suggested to play a role as possible receptors (109, 241). More recently, it was reported a novel process, integrin-using viruses bind the α2I domain of $\alpha 2\beta 1$ via DGE motif in VP4 and interact with $\alpha X\beta 2$ (via GPR motif) and $\alpha V\beta 3$ by using VP7 to facilitate cell entry and infection (105). Characterization of three rotavirus strains, which have different requirements to bind initially to and thus infect the host cell: the NA-sensitive simian rotavirus RRV, its a NA-resistant variant nar3 (185), and human rotavirus strain Wa, which is naturally resistant to NA-treatment of cells (10, 186).

Replication of the genome

Analysis of the gene coding assignements for each of the 11 genes established that there are six structural and six non-structural proteins in SA11 strain (188). The proteins of SA11 have been studied more thoroughly than those of other rotaviruses, in part because this virus was among the first of this group of agents to be propagated efficiently in cell culture (166). RNA segment 1, 2 and 3 of SA11 encodes a core or subcore structural protein, designated VP1, VP2 and VP3, respectively, whereas segment 6 encodes the major inner capsid protein VP6. Segments 4 and 9 encode the outer capsid structural proteins VP4 and VP7, respectively (179). The six non-structural proteins, which are found in infected cells but not in virions, are encoded by segments 5, 7, 8, 10 and 11 and are designated as NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6.

The nucleotide sequence of all 11 rotavirus RNA segments is known in different strains as SA11, human KU, bovine RF. Each positive-sense RNA segment starts with a 5'-guanidine followed by a set of conserved sequences 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, which contains a subset of conserved terminal 3' sequences and which ends with two 3'-terminal cytidines, is found. Almost all

mRNAs end with the consensus sequences 5'-UGUGACC-3', and these sequences contain important signals for gene expression and genome replication. 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 posses at least one long ORF after the first initiation codon. This is usually a strong initiation codon based on Kozak's rules (145). Although some of the genes posses additional in-phase (genes 7, 9 and 10) or out-of-phase (gene 11) ORFs, current evidence indicates that all the genes are monocistronic, except gene 11 in SA11 strain (181). The last 4 nucleotides of the mRNAs can function as translation enhancers (51).

The rotavirus gene sequences are A+U rich (58% to 67%), and this bias against CGN and NCC codons is shared with many eukaryotics and viral genes. The dsRNA segments are base-paired end to end, and the positive-sense strand contains a 5'cap sequence $m^7GpppG^{(m)}GPy$ (119, 182). Similar features of the RNA termini (capped structures and 5'- and 3'-conserved sequences) are found in the primary structures of the genome segments of other viruses (e,g., reovirus, cytoplasmic polyhedrosis virus, orbivirus) in the *Reoviridae* and in other virus families with segmented genomes (*Orthomyxoviridae*, *Arenaviridae* and *Bynyaviridae*). One of the most intriguing aspects of rotaviruses and all segmented dsRNA viruses relates to how these viruses co-ordinately replicate and package the 11 viral mRNAs. The 11 mRNAs must share common cis-acting signals because they are replicated by the same polymerase, and these signals are likely to be formed by secondary structures rather than the primary sequences. In addition, each mRNA must also contain a signal that is unique to it alone because the 11 mRNAs must be distinguished from one another during packaging. Generally, the conserved terminal sequences in genome segments contain cis-acting signals important for transcription, RNA translation, RNA transport, replication, assembly, or encapsidation of the viral genome segments. Some of the cis-acting signals for rotavirus RNA replication and translation have been identified, but assembly or encapsidation signals remain unknow (214).

Cis-acting elements on mRNA templates.

Examination of template mRNAs containing deletions, truncations and site-directed mutations in the replication system has allowed for the localization of *cis*-acting elements that are required for replication (210, 293). These studies, examined segment 9 (VP7) of OSU and segment 8 (NSP2) of SA11, identified a tripartite cis-acting replication signal on the respective templates. The size and locations of these signals were virtually identical on the two templates, suggesting that similar size and location signals can be expected on all 11 rotavirus mRNAs. The minimal promoter of the (-)-strand synthesis is necessary and sufficient to confer replication competence on the foreign RNA, if it is present at the 3'-terminus of that template. Immediately upstream of the promoter lies the 3'-enhancing sequence of some 25-30 nucleotides that significantly increases the activity of the minimal promoter. At the 5'-terminus of the template in the region of the nucleotide 1-10, is the 5' enhancing sequence that significantly increases template activity of templates with both the complete 3'-enhancing sequence and the minimal promoter. Interestingly, the minimal promoter and the 5'-enhancing sequence are identical to the regions at the termini of the template that are conserved on all rotavirus mRNAs and genome segments. The 3'-enhancing sequence contains the only segment-specific sequence within the *cis*-acting replication signal. Deciphering the function of each of the *cis*-acting signals is an active area of research that may provide information relevant to the development of a reverse genetic system.

One of the long-standing problems in rotaviruses, and other viruses with segmented genomes, is the mechanism by which segments are chosen from among the pool of intracellular RNAs for packaging and replication, so that a virus contains at least one copy of each genome segment. Clearly, the process is not random, the identification of *cis*-acting replication signals on template RNAs suggest that an answer to this question may be imminent. In the tripartite cis-acting signal identified on two of the genome segments, only one portion of the signal, the 3'-enhancer sequence, lies in segment-specific sequences (210, 293). This signal may provide the specificity necessary for assortment of RNAs during

genome replication and viral morphogenesis. Although the function of the 3'-enhancer sequence as the assortment signal remains to be demonstrated, the segment-specific nature of this sequence provides a model that can be tested.

Transcription and translation of proteins.

Transcription in rotaviruses, as in other members of the *Reoviridae*, is conservative; all transcripts are synthesized *de novo* and represent precise end-to-end copies of the plus strands from the eleven-genome segments (183). In addition to directing the synthesis of the viral proteins, the mRNA transcripts also serve as templates for minus strand synthesis during genome replication later in the viral life cycle. In this way, genome transcription and genome replication may be thought of as complementary processes and in fact appear to use much of the same enzymatic machinery. However, no reconstitution system is yet available for any of the reoviruses, studies conducted using baculovirus-expressed recombinant rotavirus-like particles containing the viral RNA polymerase co-expressed with the inner capsid protein have begun to clarify the mechanism by which the dsRNA genome is synthesized from mRNA templates in rotavirus (50, 210, 304). In addition, to full-length mRNA transcripts, several members of the *Reoviridae* also produce significant quantities of short oligonucleotides, which correspond in sequence to the extreme 5'-end of the mRNA transcripts and likely represent prematurely terminated transcription products. These shorter oligonucleotides are also present in both mature and transcriptionally competent rotavirus particles (151).

In rotavirus, the transcriptionally competent form of the virus has a double-layered capsid consisting of the structural proteins VP2 and VP6 surrounding the dsRNA genome segments and the enzymatic machinery of the core. Not only is the RNA-dependent RNA polymerase unable to synthesize RNA efficiently unless associated with the inner capsid protein VP2 (213), but the intermediate VP6 capsid layer must also be present (22, 143, 249) and the outermost VP7 capsid layer absent (49, 56, 151). These observations have led to the proposal

that the functional endogenous transcription apparatus contains three components: a) the enzymes required for RNA synthesis and capping, b) a scaffold consisting of the intact VP2 capsid layer functionally enhanced by the presence of VP6 on the exterior surface, and c) the viral nucleic acid (230).
The process of the viral mRNA synthesis may be considered to involve three distinct events:a) *initiation*, in which the polymerase complex and the nucleotides at the 3' end of the minus strand interact in a manner that allows nucleotidyl transfer and capping to occur; b) *elongation*, in which nucleotidyl transfer progresses as the polymerase complex moves along the helical axis relative to the dsRNA template; and c) *translocation*, in which the growing mRNA transcript is transported across the intact capsid trough specific channels to exit the particle. Inititation and elongation primarily involve enzymatic components of the transcription apparatus and occur in the viral core, whereas translocation primarily involves the capsid region of the virus (150).

Rotavirus architecture

Mature, infectious rotaviruses have a multilayered capsid structure (230, 231, 298). The inner most capsid layer is formed by 120 molecules of a 102 kDa protein VP2 arranged as 60 dimers on T=1 icosahedral lattice (152). Such an organization with two molecules in the icosahedral asymmetric unit has also been referred to as 'T=2'structure (107). This protein shell is believed to define the basic icosahedral architecture of the virus, as VP2 is the only rotavirus protein capable of self assembling into stable virus–like particle (VLPs) (58, 148). The VP2 layer serves as a scaffold for the assembly of the intermediate layer, which consists of 780 copies of VP6 arranged as 260 trimers on a T=13 (*levo*) icosahedral lattice. The addition of VP6 protein to VLPs formed from VP2 imparts greater morphological homogeneity and long-term stability to the particles (304), suggesting that VP6 may play a major role in providing structural integrity rotavirus capsid. The icosahedral organization of the VP6 capsid layer defines a set of channels leading to the viral interior; these channels play an important role

in the transcription process, facilitating the import of RNA precursor molecules and the export of mRNA transcripts (231). The outer-most capsid layer in the mature particle consists of 780 copies of VP7 assembled in a 1:1 stoichiomestry onto the top of each VP6 molecule. The outer capsid layer also contains 60 dimers of VP4, which form spikes extending 120 Å outward from the viral surface (229, 256, 297).

The atomic resolution structure of the VP6 capsid protein of rotavirus was recently determined using X-ray crystallographic techniques (175). The rotavirus VP6 folds into distinct domains of roughly equal size. The upper domain, which interacts with VP7 in the mature particle, fold into an eight- stranded β-barrel structure, a motif often seen in viral capsid proteins (figure 6).

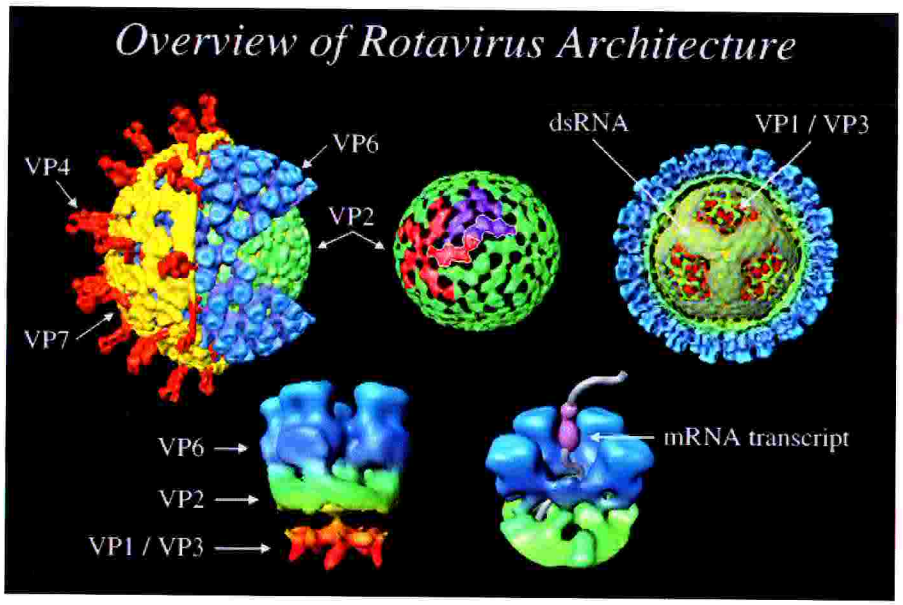


Figure 6.: a) 3D reconstruction of a mature, triple-layered rotavirus particle. Image obtained by cryoelectron microscopy. Part of the middle and outer protein layers were cut away to show the inner structure of the virus. b) When represented at an elevated contour threshold (~ 2.3σ), the surface appears to separate into 60 antiparallel strips of mass density, with each strip extending from one icosahedral vertex (5-fold axis) to a point near and adjacent vertex. One of these as such, may be a dimmer of quasequivalnet VP2 molecules. These two types of VP2 are designated as A (red) and B (purple). c) The transcription enzymes VP1 and VP3 form a complex that is anchored to the inner surface of the VP2 capsid layer. In this croos-sectional illustration produced from a 3D reconstruction of 1/3/2/6-VLP (230) and shown oriented along the icosahedral vertex. d) The VP1/3 complex appears as a flower-shaped structure (orange) attached to a small inward prostrusion of a scaffold for the enzymatic and nucleic acid components of the transcription of VP2 a the 5-fold axis. e) During transcription in rotavirus, capped mRNA transcripts corresponding to the 11 dsRNA genome segments are synthesized in the viral core and then translocated 140 Å trough a system of channels penetrating the two capsid layers at the icosahedral vertices (150).

Virus release

Rotavirus undergoes a unique maturation process. Immature subviral particles assemble in cytoplasmic viroplasms structures, bud trough the endoplasmic reticulum (ER) membrane, and acquire a transient envelope (216, 217). Subsequently, this membrane is lost (274), and the outer capsid protein VP7, retained in ER, is folded into the subviral particles to form the definitive, mature viral particles (72). Studies using MA104 cells, rotaviruses were known to be retained in the ER lumen until cell lysis (6, 199). However, this nonpolarized cell does not display any morphologic or functional characteristics of rotavirus natural target cell. Moreover, viral spreading through cell lysis does not match recent data indicating that rotavirus may infect and multiply during more than one cycle of replication, without cell death or membrane leaks (265). This observation correlates with *in vivo* data demonstrating that rotavirus may replicate without any histopathologic changes (36, 273). The polarized intestinal CaCO-2 cells, has been shown to spontaneously display many of the morphologic and biochemical properties of mature enterocytes. In these cells, rotavirus was released almost exclusively at the apical pole before any cells lysis was detected. The rotavirus transport pathway from the ER to the apical surface of the intestinal cells describe a nonconventional vesicular transport of rotavirus particles from the ER to the apical plasma membrane, that bypasses the Golgi apparatus and lysosomes (129).

Role of host cell during viral infection

In non-polarized cells, the rotavirus infection present a evident cytophatic effect, and cells death is preceded by the shuthoff of host RNA, DNA and protein synthesis (41, 70, 182).

Upon infection of nonpolarized cells, rotavirus induces early alterations in vimentin (289), in the cytoskeleton organization (290), and in biochemistry of the host cell (183). A dramatic decrease in the level of host cell proteins and an increase of viral proteins is observed (70). Four proteins that are specifically up-

regulated in rotavirus–infected cells have been identified, two of which correspond to the ER chaperones BiP and endoplasmin; however, the role of these proteins in the replication and morphogenesis of rotavirus particles remains to established (296).

Rotavirus infection in polarized cells as CaCO-2 or MDCK shown that rotavirus infects polarized cells efficiently through the apical surface. Additional studies have shown that rotavirus infection induces microvillar F-actin disassembly (128), and an increase in $[Ca^{2+}]_i$ is associated with release of a viral protein or peptide from infected cells that induces a Ca^{2+}-dependent microvillar F-actin alteration in uninfected CaCO-2 cells (37). In addition, rotavirus infection causes alterations in tight junctions independent of virus-induced cytosketeletal rearregements (205). Both, MDCK and CaCO-2, infected cells show transmembrane leaks or opening of tight junctions before the development of cytopathic effect and extensive virus release. The epithelial cell response to rotavirus infection has also been examined in nonpolarized cells lines (43, 257). Interleukin-8, growth-related peptide-alpha, and RANTES are secreted from rotavirus–infected cells, and chemokine production is time and dose dependent. Interferons and GM-CSF have also been detected in mice infected with rotavirus (240). The chemokine secretion response to rotavirus infection play a role in viral pathogenesis and in the immune response to rotavirus infection.

Finally, rotavirus replication in neuronal cells was studied. In this system, the expression of VP7 is targeted to axons and dendrites by a pathway that does not involve the Golgi apparatus (288). In constrast, NSP4 is localized solely in the cell body. Rotavirus infection stimulates alterations of specific neuronal proteins. Thus, the distribution of microtubule-associated protein 2, which is normally restricted to nerve cell bodies and dendrites, is altered and found in axons of cultured dorsal root ganglia and spinal cord neurons. It is not known if rotaviruses naturally infect neurons or if such infections might result in activation of the enteric nervous system, which is reported to be component of the pathogenesis of diarreal disease (72, 162).

Role of phosphorylation in virus

Due that in this thesis, the role of the phosphorylation is extensively studied in the NSP5 context. It is important to full understand this problematic, define a series of concepts as kinase classification and the role of the phosphorylation in the viral environment. The protein kinases are defined with general property of catalysing the transfer of γ-phosphate of a nucleoside triphosphate to a hydroxyamino acid residue of a protein substrate. The nucleoside triphosphate is generally ATP, although in a few cases, most notably that of the enzyme know as casein kinase 2 and its apparent nuclear equivalent, both ATP and GTP can be phosphate donors. The hydroxyamino acceptor can be serine, serine or threonine, or tyrosine depending on the particular protein kinase (153). Protein kinases mediate most of the signal transduction in eukaryotic cells; by modification of substrate activity, protein kinases also control many other cellular processes, including metabolism, transcription, cell cycle progression, cytoskeletal rearregement and cell movement, apoptosis, and differentiation. Protein phosphorylation also plays a critical role in intercellular communication during development, in physiological responses and in homeostasis, and in the functioning of the nervous systems. Protein kinases are among the largest families of genes in eukaryotes and have been intensively studied. Most protein kinases belong to a single superfamily containing an eukaryotic protein kinase (ePK) catalytic domain. To compare related kinases in human and model organisms and to gain insights into kinase function and evolution, the kinases were classified into a hierarchy of groups, families, and subfamilies. This extends to Hanks and Hunter human kinase classification of five broad groups, 44 families, and 51 subfamilies by adding four new groups, 90 families, and 145 subfamilies. Kinases were classified primarily by sequence comparison of their catalytic domains, known biological functions, and a similar classification of yeast, worm and fly kinomes. Four new groups were added to the list, STE consists of MAPK cascade families (Ste7/MAP2K, Ste11/MAP3K, and Ste20/MAP4K). The CK1 group contains CK1, TTBK (tau tubulin kinase), and VRK (vaccinia-related kinase) families. TKL (tyrosine kinase-like) is a diverse

group of families that resemble both tyrosine and serine-threonine kinases. It consists of MLK (mixed-lineage kinase), LISK (LIMK/TESK), IRAK [interleukin-1 (IL-1) receptor associated kinase], Raf, RIPK [receptor-interacting protein kinase (RIP)], and STRK (activin and TGF-β receptors) families. Members of the RGC (receptor guanylate cyclase) group are also similar in domains sequence to tyrosine kinase (168).

When cells infected with a wide variety of viruses are incubated with [^{32}P] orthophosphate, certain viral proteins become radioactively labelled. The animals viruses for which phosphoproteins have been reported include members of the following classes: *Herpesviridae*, *Adenoviridae*, *Papovaviridae*, *Hepdnaviridae*, *Poxviridae*, African swine fever virus *Parvoviridae*, *Picornaviridae*, *Togaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Orthomyxoviridae* and *Retroviridae*. It has been demonstrated in only a few cases that alterations in the functional properties of the viral proteins can occur as a consequence of there being phosphoproteins. All these appear to involve effects on the interaction of the phosphoprotein with nucleic acid, a type of functional modulation with few understood precedents from non-viral systems. It is necessary to draw a distinction between the functional significance and regulatory significance that the phosphorylation of a viral protein might have. The phosphorylation of a particular viral enzyme would have functional but not regulatory significance if it were required for the catalytic activity of the enzyme, but occurred immediately after the synthesis of the enzyme and persisted throughout its lifetime. Such a constitutive functional phosphorylation might be envisaged as occurring where the simpler evolutionary alternative, a genetically coded acidic residue, is inadequate. A phosphorylation would have both functional and regulatory significance if, for example it occurred late in infection, causing inactivation of an enzyme which was synthesized and active early in infection (153).

A series of examples, in which phosphoproteins of different viruses have variable functions. Some of these are: a) the virus membrane protein A14 from vaccinia virus in which, repression of this protein results in a 1000 fold reduction in viral yield, leads to an early block in viral morphogenesis characterized by a large

accumulation of large virosomes, empty "crescents" that fail to contact these virosomes and most strinkingly, large numbers of aberrant 25 nm vesicles (187); b) also in Chandipura virus, the phosphoprotein P, besides nucleocapsid protein N, acts as a leader RNA-binding protein in its unphosphosphorylated form, whereas CK2-mediated phosphorylation totally abrogates its RNA-binding ability (17); c) Other viral phosphoproteins are able to interact with cytoplasmic proteins as the HCV non-structural protein 5A (NS5A) protein that as been shown associated with a wide variety of cellular signalling proteins. Of particular interest is the observation that a highly conserved C-terminal class II polyprolyne motif with NS5A mediated association with Src homology 3 domains of members of Src family of tyrosine kinases and the mitogenic adaptor protein Grb 2 (164). In conclusion a many extensive series of functions can be associates to the phosphoproteins. For which each phosphoprotein should be analyzed to find their function whether structural or in the regulation of the replication of viruses.

MATERIALS AND METHODS

Tissue culture

MA104 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) (Gibco-BRL) and gentamicin (100 µg/ml).

BSC-40 cells were routinely cultured in DMEM supplemented with 10% bovine calf serum (BCS) (Keystone) and gentamicin (100 µg/ml).

Stable transfected cells (C7) were cultured in DMEM supplemented with 10% foetal calf serum, geneticin (G-418) 500 µg/ml (Gibco-BRL) and gentamicin (100 µg/ml).

Cells were propagated using tripsin (500 µg/ml) and inhibited with complete medium. The cells were centrifugated at 1000 rpm for 2 min and resuspended in complete medium

Virus propagation

The simian rotavirus strain SA11 and porcine rotavirus strain OSU were propagated and grown in MA104 cells as described (75).

The vaccinia recombinant virus induced by IPTG, VT7/LacOI/NSP2, were propagated and grown in BSC-40 cells as described by Ward *et al*, 1995 (286).

The T_7 RNA polymerase recombinant vaccinia virus, strain vTF7.3 was propagated in HeLa cells as described by Fuerst *et al*, 1986 (88).

Constructs preparation

The constructs pT_7v-NSP5, pT_7v-Δ1, pT_7v-Δ2, pT_7v-Δ3, pT_7v-Δ4, pT_7v-ΔT, pT_7v-Δ1/Δ2 and pT_7v-Δ4T, were previously described by (4, 77). Internal deletion mutants were obtained by PCR using specific internal primers for the construction of pT_7v-Δ1/Δ4T, pT_7v-Δ1/Δ3, pT_7v-4T, pT_7v-Δ2/Δ4T, pT_7v-Δ2/Δ3, pT_7v-Δ3/ΔT, pT_7v-Δ1/Δ3T and pT_7v-Δ1/ΔT; and cloned as KpnI/BamHI fragments in pcDNA3 (Invitrogen).

pT_7v-His_6-Δ1/Δ3 and pT_7v-His_6-Δ1 were obtained by inserting at the 3'-end the His_6 tag with oligonucleotides, 5'-AGCTTGTACCATGGGTCATCAC

CATCACCATCATGGTAC-3' and 5'-CATGATGGTGATGGTGATGA CCCATGGTACA-3', into the multiple cloning site of the vector pcDNA3 with the restriction enzymes HindIII/KpnI. The pT7v-His_6-Δ1/Δ3 (S→A) and the serine mutants [Δ1/Δ3(S153/5A), Δ1/Δ3(S155A), Δ1/Δ3(S153/5-163), Δ1/Δ3(S155-163/5), Δ1/Δ3 (S163A), Δ1/Δ3 (163/5A) and Δ1/Δ3(153/5,163/5)] were obtained by double step PCR using internal oligonucleotides. These were cloned as KpnI/BamHI fragments into pT_7v-$(His)_6$.

pT_7v-Δ3a, pT_7v-Δ3b, pT_7v-Δ3ab, pT_7v-Δ3/S63A, pT_7v-Δ3/S65A, pT_7v-Δ3/S67A and pT_7v-Δ3(S63,65A/S67D) were obtained by double step PCR using internal oligonucleotides to amplified regions 1 and 2 and cloned as a KpnI/ClaI fragments in pT_7v-(4TclaI5'). pT_7v-(4TclaI5') was obtained by PCR of the regions 4 and T, inserting the restriction sites KpnI and ClaI at the 5'-end, and BamHI at the 3'-end. The fragment was cloned as KpnI/BamHI in pcDNA3.

pT_7v-Δ3c was amplified by PCR using as template the regions 1 and 2 to insert KpnI and ClaI restriction sites and cloned KpnI/ClaI in pT_7v-[4T(S→A)claI5']. pT_7v-[4T(S→A)claI5'] amplification of regions 4 and T from pT7v-His_6-Δ1/Δ3 (S→A) to insert restriction sites KpnI/ClaI at the 5'-end, and BamHI at the 3'-end. The fragment was cloned as KpnI/BamHI in pcDNA3.

pT_7v-SV5Δ2 , pT_7v-SV5Δ4 and pT_7v-SV5Δ3c were obtained by inserting at the N-terminus the SV5 tag with oligonucleotides, 5'-AGCTTGTACCATG GGCAAACCAATCCCAAACCCACTGCTGGGTCTGGATGGTAC-3' and 5'-CATCCAGACCCAGCAGTGGGTTTGGGATTGGTTTGCCCATGGTACA-3', and into HindIII/KpnI.

PT_7v-NSP5a, pT_7v-NSP5/S67A and pT_7v-NSP5(S63,65A/S67D) were amplified by PCR of the respective pT7v-Δ3 point mutation, in order to use specific primers to incorporate KpnI and BstBI restriction sites at the 5' and 3' ends of the 1+2 region, respectively. The fragments were cloned KpnI/BstBI into pT7v-Δ1Δ2(KpnI/BstBI). These constructs were obtained by PCR with specific primer to insert KpnI/BstBI and BamHI restriction sites at the 5' and 3' ends of the Δ1Δ2 region, respectively.

GST-NSP5 was obtained by cloning a BamHI fragment of NSP5 in pGex2T (Pharmacia). Similarly, GST-Δ1, -Δ2, -Δ3, -Δ4, -ΔT, -Δ4T, -Δ1/Δ3, -4T and Δ1/Δ3(S→A) were obtained by cloning the respective fragments (KpnI/BamHI) in a modified pGex2T vector to include the BamHI and KpnI sites.

Constructs pEGFP-NSP5, pEGFP-Δ1, pEGFP-Δ2, pEGFP-Δ3, pEGFP-Δ4, pEGFP-Δ1/Δ3, pEGFP-ΔT were obtained by PCR using as template from previously described constructs (4, 77) using specific primers to incorporate EcoRI and a PstI restriction sites at the 5'- and 3'-ends, respectively. Similar oligonucleotides were used to construct pEGFP-4T, pEGFP-Δ1/Δ4T and pEGFP-Δ4T. All these fragments were cloned as EcoRI/PstI fragments in pEGFP-N1 (Clontech).

pEGFP-Δ1/Δ3(S→A) was obtained by insertion of a linker sequence in pEGFP-N1 (5'-AATTCCTGGTACCACACTGCAGGTAAGG-3' and 5'-GATCCCTTA CCTGACGTGTGGTACCAGG-3') followed by cloning of a fragment KpnI/PstI from pT_7v- His_6-Δ1/Δ3 (S→A). Similarly, pEGFP-Δ3a, pEGFP-Δ3b, pEGFP-Δ3ab, pEGFP-Δ3/S67A and pEGFP-Δ3(S63,65A/S67D) were obtained by subcloning of pT_7v-Δ3a, pT_7v-Δ3b, pT_7v-Δ3ab, pT_7v-Δ3/S67A and pT_7v-Δ3(S63,65A/S67D) fragments digested KpnI/PstI in pEGFP-N1 modified polylinker.

pEGFP-NSP2 was obtained by the insertion of the fragment NSP2 in a pEGFP-N1 with KpnI/BamHI restriction sites. The p(1-EGFP-4T),p(1-EGFP-T), p(1-EGFP) and p(EGFP-4T) were obtained by insertion of the region 1 with EcoRI/BamHI restriction sites in the 5'-end and the regions 4 and tail with BsrGI/NotI restriction sites in the C-terminal of EGFP in the pEGFP-N1 (Clontech) vector. The pT_7v-(dom1EGFP4T), pT_7v-(dom1EGFPT), pT_7v-(dom1EGFP), pT_7v-(EGFP4T) and pT_7v-(EGFPT) were obtained by subcloning with EcoRI/ NotI from the previous described constructs in pcDNA3.

The construct pVOTE.1/NSP2 was obtained by PCR from the previous described pT_7v-NSP2 (4) by using specific primer to incorporate *Nco*I and *Bam*HI restriction sites at the 5'- and 3'-ends, respectively. The fragment was cloned NcoI/ BamHI in pVOTE.1 (286).

For the two hybrid bait constructs pBMT-NSP5, pBMT-Δ2, pBMT-Δ4, pBMT-ΔC48, pBMT-Δ1/Δ3, pBMT-Δ2/Δ3 were obtained by PCR from the constructs described above by using specific primers to incorporate *Eco*RI and *Bam*HI restriction sites at the 5'- and 3'-ends, respectively. All these fragments were cloned as EcoRI/BamHI fragments in pBMT116 (283). The fish pV16/D(NSP2) was obtained by inserting the oligonucleotides 5'CGCGCGCATATGGCTGAG3' and 5'CTAGCTCACCATATGCGCGCGGTAC3' at the N-terminal and the oligonucleotides 5'-GATCCGTACTCTAGAG-3' and 5'-TCGACTCTAGAGTACG-3' at the C-terminal, to generate a *Bss*HII and an XbaI restriction sites in pT_7v-NSP2, respectively (4).The fragment was cloned *Bss*HII/*Bam*HI in pVP16/D, that is a modified vector of pVP16*(283).

Oligonucleotides

The internal primers used and oligonucleotides for the construction of the different deleted mutants of NSP5 and for the construction of the different plasmids, are signalled in table 2.

Table 2. Oligonucleotides used for the different constructions

construction name	oligonucleotides sequence[a]
pT_7v-Δ1/Δ3	5'-AATGGTACCATGATTGGTAGGAG-3'
	5'-GATCAGCGAGCTCTAGC-3'
pT_7v-4T	5'-CGGGGTACCATGGATAATAAAAAGGAG-3'
	5'-GCGGGATCCTTACAAATCTTCGATC-3'
pT_7v-Δ1/Δ4T	5'-AATGGTACCATGATTGGTAGGAG-3'
	5'-TTACTGCAGAGTTGAGATTGATAC-3'
pT_7v-Δ2/Δ4T	5'-CGGGGTACCATGTCTCTCAGC-3'
	5'-CGCGGATCCTTAAGTTGAGATTGAT-3'
pT_7v-Δ3/ΔT	5'-CGGGGTACCATGTCTCTCAGC-3'
	5'-CGCGGATCCTTAGTACTTTTTCTTA-3'
pT_7v-Δ2/Δ3	5'-CGGGGTACCATGTCTCTCAGC-3'
	5'-GCGGGATCCTTACAAATCTTCGATC-3'
pT_7v-His_6-Δ1/Δ3(S→A)	5'-TCAGCATCTGCATCATCTAAAACATAATCTTC-3'
	5'-GATGCTGACGCTGAAGATTATGTTTTAGATGA-3'.
pT_7v-Δ3ab	5'-GAGGATATTGGACCAGCAGATGCTGCTGCAAAC
	GATCCACTAACCGCATTTGCTATTAGAGCTAATGCA-3'

pT_7v-Δ3a	5'-GAGGATATTGGACCAGCAGATGCTGCTGCAAAC
	GATCCACTAACCAGCTTTTCGATTAGATCGAATGCA-3'
pT_7v-Δ3b	5'-AACGATCCACTAACCGCATTTGCTATTAGAGCT
	AATGCA-3
pT_7v-Δ3/S63A	5'-GAGGATATTGGACCAGCAGATTCTGCTTCAAAC
	GATCCACTAACCAGCTTTTCGATTAGATCGAATGCA-3'
pT_7v-Δ3/S65A	5'-GAGGATATTGGACCATCTGATGCTGCTTCAAAC
	GATCCACTAACCAGCTTTTCGATTAGATCGAATGCA-3'
pT_7v-Δ3/S67A	5'-GAGGATATTGGACCATCTGATTCTGCTGCAAAC
	GATCCACTAACCAGCTTTTCGATTAGATCGAATGCA-3'
pT_7v-Δ3(S63,65A/S67D)	5'-GAGGATATTGGACCAGCAGATGCTGCTGACAAC
	GATCCACTAACCAGCTTTTCGATTAGATCGAATGCA-3'
pT_7v- SV5Δ2(S137/142A), SV5Δ2c(S137/142A),	5'-GATAATAAAAAGGAGAAAGCAAAGAAAGATAAAGCT
	AGGAAACACTACCCGAGA-3'
pT_7v- SV5Δ2(S137A), SV5Δ2c(S137A)	5'-GATAATAAAAAGGAGAAAGCAAAGAAAGATAAAAGT
	AGGAAACACTACCCGAGA-3'
pT_7v- SV5Δ2(S142A), SV5Δ2c(S142A)	5'-GATAATAAAAAGGAGAAATCCAAGAAAGATAAAGCT
	AGGAAACACTACCCGAGA-3'
pEGFP-Δ1, Δ1/Δ3	5'-CGGGAATTCATGATTGGTAGGAG-3'
	5'-GATCCTTACTCGAGCAAATCTTCGATCAATTGCA-3'
pEGFP-ΔT	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-TTACTGCAGGTACTTTTTC-3'
pEGFP-4T	5'-CGGGAATTCATGGATAATAAAAAGGAGAAATCC-3'
	5'-GATCCTTACTCGAGCAAATCTTCGATCAATTGCA-3'
PVOTE1.NSP2	5'-GATCCGTAGTCTAGAG-3'
	5'-TCGACTCTAGAGTACG-3'
pBMT-NSP5	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-GCGGGATCCTTA CAAATCTTCGATC-3'
pBMT-Δ2	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-GCGGGATCCTTA CAAATCTTCGATC-3'
pBMT-Δ4	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-GCGGGATCCTTA CAAATCTTCGATC-3'
pBMT-Δ2/Δ3	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-GCGGGATCCTTA CAAATCTTCGATC-3'
pBMT-Δ1/Δ3	5'-CGGGAATTCATGATTGGTAGGAG-3'
	5'-GCGGGATCCTTACAAATCTTCGATC-3'
pBMT-ΔC48	5'-CCGGAATTCATGTCTCTCAGCATTG-3'
	5'-TGATCAGCGAGCTCTAGC-3'

[a] primers are listed as forward and reverse pairs.

Transient transfection of MA104 cells and cellular lysis

To prepare cellular extracts, cells were transfected essentially as described by Afrikanova *et al.* 1998 (4). Briefly, 5 x 10^5 cells growing on a 35 mm diameter Petri dish, were infected for 1 h with T7-recombinant vaccinia virus (strain vTF7.3) (88) and then transfected with 5 μl of Transfectam reagent (Promega) containing 2 μg of plasmid DNA and incubated for 16 h.

At 16 h post- transfection cells were washed twice with PBS, lysed in 60 μl of TNN lysis buffer (100 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5 % NP40 and 1 mM PMSF) for 10 min at 4ºC and centrifuged at 10000 x g for 5 min. Supernatants were used as cellular extracts in the kinase assays.

Transient transfection with EGFP fusion proteins for immunofluorescence, cells were grown on a 35 mm diameter Petri dish, transfected with 7.5 μl of Transfectam reagent containing 5 μg of plasmid DNA in DMEM serum free. After 6 hours post-transfection, medium was changed by DMEM, 10% FCS and incubated for 48 h. Immunofluorescence was performed as described below.

Stable transfection with calcium phosphate

The cell were transfected with calcium phosphate essentially as described by Sambrook et al., 1989 (247). 1.5 x 10^6 cells were platted in 100 mm diameter Petri dish. Fresh medium was added 4 h before transfection. 6 μg of linearised plasmid DNA was resuspended in 50 μl of 0.1 X TE (10 mM Tris, 1 mM EDTA). Mix A was prepared by addition of 169 μl of deonized water, then 5 μl $CaCl_2$ 2 M, next added drop by drop DNA, and 26 μl $CaCl_2$ 2 M. All was mixed slowly for two times. The mix A was added in mix B containing 250 μl 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $NaHPO_4$, 12 mM dextrose and 50 mM Hepes). Total mix was added at cells drop by drop. The cells were incubated O.N. and the medium was changed by complete medium supplemented with 500 μg/ml G-418. The cellular foci were chose after one week on selective medium.

Immunofluorescence and antibodies preparation

For indirect immunofluorescence microscopy cells were fixed in 3,7% paraformaldehide in PBS for 10 min at room temperature. Cover slips were

dehydrated in PBS and blocked with 1% BSA in PBS for 30 min and incubated with guinea-pig anti-NSP2 serum (1:100) in PBS-1% BSA for 1 h in moist chamber at room temperature. After three washing in PBS, slides were stained for 45 min with RITC-conjugated goat anti-guinea pig antibody (Sigma), washed and mounted with ProLong mounting medium (Molecular Probes). Samples were analysed by confocal microscopy (Axiovert; Carl Zeiss).

For double immunofluorescence for NSP5 and NSP2, cells were incubated firstly with mouse anti-NSP2 (1:100) in PBS-BSA 1% for 1 h in moist chamber at room temperature. The second incubation was performed with a guinea pig anti-NSP5 (1:100) in PBS-BSA 1% for 1 h in moist chamber at room temperature. The third incubation is a mix of RITC-conjugated goat anti-guinea pig antibody (Sigma)(1:100) and FITC-conjugated goat anti-mouse antibody (Dako)(1:100) in PBS-BSA 1%. The fixation, dehydratation, washing and mounting are as described above.

Anti-NSP5 and anti-NSP2 sera were obtained by immunisation of guinea pigs and mice with GST-NSP5 or GST-NSP2 fusion proteins essentially as described (102).

Western blot

Samples were loaded in a SDS-PAGE (149). After electrophoresis, proteins were transfered to a PDVF membrane (Immobilion-P), for 2 h at 200 mA or O.N. at 50 mA. The membrane was blocked in PBS-milk 5% for 30 min and incubated for 1 h with the primary antibody in PBS-milk 5%. The membrane was then washed three times with PBS-milk 5%, and incubated for 1 h with the secondary antibody conjugated to horse radish peroxidase (HRP). Finally, the membrane was washed 3 times in PBS-milk 5% for 5 min and once in PBS. The membrane was developed using the ECL kit (Pharmacia). The dilutions used for the primary antibodies and for the secondary antibodies are indicated in table 3.

Table 3. Antibodies for WB and dilutions

First antibody	Secondary antibody[a]
Guinea pig anti-NSP5 (1:3000)	Goat anti-guinea pig-HRP (1:2500)(KPL)
Guinea pig anti-NSP2 (1:3000)	Goat anti-guinea pig-HRP (1:2500)(KPL)
Mab anti-SV5 (1:5000)	Goat anti-mouse-HRP (1:5000)(KPL)
Rabbit anti-LexA (1:5000) (Invitrogen)	Goat anti-rabbit-HRP(1:5000)(KPL)
Rabbit anti-VP16(1:200) (Clontech)	Goat anti-rabbit-HRP(1:5000)(KPL)

[a] the dilutions for the secondary antibodies are the recommended by distributor

Purification of $(His)_6\Delta1/\Delta3$

Transfected cellular extracts were incubated for 1 h at 4°C with nickel beads, NTA-agarose (Amersham Pharmacia Biotech) and equilibrated in 5 volumes of loading buffer (20 mM imidazole, 5 mM DTT in PBS). Beads were then washed with 10 volumes of washing buffer (35 mM imidazole, 5 mM DTT in PBS) and once with 35 mM imidazole, 400 mM NaCl, 5 mM DTT in PBS. The protein was eluted with 2 volumes of elution buffer (250 mM imidazole, 0.02% sodium azide, 5 mM DTT in PBS) and dialysed against in PBS containing 5 mM DTT. The recovered protein was analysed by Western blot using guinea pig anti-NSP5 serum.

Expression of GST fusion proteins

GST fusion proteins were produced in *E. coli* DH5α. Cultures were induced with 3 mM IPTG for 3-4 h at 37ºC. The bacteria was centrifuged and the pellet washed with ice cold PBS and resuspended in 1,5% laurylsarcosinate-PBS with the adding of 0,1 μg/μl lysozime, 0,1 μg/μl CLAP and 5 mM DTT, for sonication (6 times, 10s). The supernatant was supplemented with 1% Triton X-100 in PBS and equilibrated with slurry beads Glutathione Sepharose 4 Fast Flow (Amersham Pharmacia Biotech). After rolling for 1 h at 4°C, the sample was centrifuged at 1000 x g for 5 min at 4°C and washed three times with 20 volumes of PBS-1%Triton X-100. Elution was performed with 2 volumes of elution buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 5 mM DTT, 0.1% Triton X-100, 50 mM reduced glutathione).

Generation of recombinant vaccinia VT7/LacOI/NSP2.

To generate the recombinant virus, BSC-40 cells were infected with the recombinant vaccinia virus VT7/LacOI and transfected with pVOTE.1/NSP2. Selection and amplification of VT7/LacOI/NSP2 was carried out as described by Ward *et al.*, 1995 (286). The plasmid vectors pVOTE.1 and the rVV VT7/LacOI were kindly provided by Bernard Moss (National Institutes of Health, Bethesda, Md).

Analysis of protein expression

MA104 cells were infected with VT7/LacOI/NSP2 at a multiplicity of infection (MOI) of 6 PFU/cell and maintained either in the presence or absence of the inducer IPTG (1 mM final concentraction). At 18 h postinfection cells were washed twice in PBS, starved for 30 min in methionine free-DMEM and metabolically labelled for 1 h with 300 μCi of [^{35}S] methionine. The cells lysis and protein analysis are described below.

VLS formation

0.5 x 10^6 MA104 or C7 cells (4) in 30 mm diameter Petri dishes were infected with 3 PFU/cell of VT7/LacOI/NSP2 for 1 h. Then, cells were transfected with 2 μg DNA plasmid and 5 μl of Transfectam (Promega). Cells were incubated for 18 h in presence of 1mM IPTG and 100 μg/ml of rifampicin in DMEM serum free. Finally, cells were fixed and processed for immunofluorescence as described above.

In vivo ^{32}P labelling

The ^{32}P labelling of 0.5x10^6 transfected cells was performed at 15 h post-transfection. Cells were washed three times in phosphate-free minimal essential medium, and then starved for 30 min in 1 ml of the same medium. Then, 30 μl of carrier free $^{32}P_i$ (Amersham Pharmacia Biotech, 10 mCi/ml) was added and incubation continued for 1h at 37ºC and lysed as described above.

In vitro translation

The *in vitro* translated proteins were synthesised essentially as described by Afrikanova *et al.*, 1996, using the TNT-T7 Coupled Reticulocyte Lysate System (Promega). Briefly, 1 μg plasmid construct was transcribed using T_7 RNA polymerase and the transcript translated in rabbit reticulocyte lysates in presence of 4 μl (1000 Ci/mmol) [^{35}S] methionine and incubated 1.5 h at 30°C.

Kinase assay

In vitro translation/phosphorylation assay

The *in vitro* translation/ phosphorylation assay was performed in a total volume of 50 μl, containing 10 μl of *in vitro* translated proteins, 15 μl cellular extract, 5 μl of kinase buffer (500 mM Tris-HCl pH8; 15 mM spermidine; 8 mM $MgCl_2$; 10 mM DTT, 5 mM ATP and 50 % glycerol). The reaction was incubated for 25 min at 37°C, stopped with 5 μl 50mM EDTA and immunoprecipitated with a guinea pig anti-NSP5 serum.

For kinase assays in presence of synthetic peptides, peptides were added at the reaction with a volume of 5 μl (10% reaction) in a range of concentration between 0 and 500 μM.

In vitro phosphorylation of bacteria recombinant protein with ^{32}P

For the assay with the GST fusions, reactions were carried out in 50 μl in the same reaction buffer containing 0.2 μg GST fusion protein, 1 μl cellular extract, and 10 μCi of [^{32}P]-γ-ATP, (3000Ci/mmol) or [^{32}P]-γ-GTP (5000Ci/mmol) (Amersham). The reaction was incubated for 25 min at 37°C, stopped with 5 μl 50mM EDTA and immunoprecipitated with a guinea pig anti-NSP5 serum.

In vitro phosphorylation of cellular extracts with ^{32}P

15 μl of cellular extract were in a reaction buffer with 500 mM Tris-HCl pH8; 15 mM spermidine; 8 mM $MgCl_2$; 10 mM DTT, 250 μM ATP and 50% glycerol and 10 μCi of [^{32}P]-γ-ATP (3000 Ci/mmol) or [^{32}P]-γ-GTP (5000 Ci/mmol) incubated in a total volume of 50 μl. The reaction was incubated for 25 min at 37°C, stopped

with 5 μl 50 mM EDTA and immunoprecipitated with a guinea pig anti-NSP5 serum.

CK2 kinase assay

For the CK2 assay substrates were incubated in total volume of 50 μl in a reaction buffer containing 50 mM Hepes pH 7.8, 10 mM $MgCl_2$, 150 mM NaCl and 0.5 mM DTT, 4 μM ATP supplemented with 1 μCi [γ^{32}P]ATP (3000Ci/mmol) and 5 units of recombinant GST-CK2α /GST-CK2β. (242). Incubation was for 25 min at 30°C. As control, 100 ng of β-casein were used per reaction. We established the correspondence between the activities of CK2 and the Δ1/Δ3 cellular extract, using as substrate the GST-Δ1 fusion: 1 μl of cellular extract corresponded to approximately 0.7 units of recombinant CK2, measured either in conditions of the *in vitro* phosphorylation assay or the CK2 assay.

Where indicated λ-phosphatase (λ-Ppase) treatment was performed on immunoprecitates in 50 μl reactions containing 50 mM Tris-HCl, pH 7.8, 5 mM DTT, 6 mM $MnCl_2$ and 2 μl λ-Ppase (400000 U/ml) (New England Biolabs) and incubated at 30°C for 2h.

In vivo hyper-phosphorylation assay

The cells were co-transfected with 1 μg of SV5-substrate and 1 μg of kinase activator NSP5 mutant in presence of T_7-recombinant vaccinia virus as described above. After 16h, cellular extracts were prepared and 15 μl of samples were loaded in a 15% SDS-PAGE. A Western immunoblotting anti-SV5 was performed.

Peptides synthesis

The peptides used in the kinase assays were synthesised chemically by Zotir Zahariev (Protein structure and bioinformatics, ICGEB).

Immunoprecipitations and PAGE analysis

Kinase reactions (50 μl) and cellular extracts (50 μl) were immunoprecitated adding 1.5 μl guinea pig anti-NSP5 serum, 1 μl 100mM PMSF, 50 μl of 50%

protein A-Sepharose CL-4B beads (Pharmacia) in TNN buffer and 60 µl TNN buffer, for 2h at 4°C. Beads were washed three times with TNN buffer and samples analysed in SDS-PAGE (149). Visualisation of [^{35}S] labelled proteins was enhanced by fluorography using Amplifiy (Amersham). Autoradiography was performed at –70°C using X-ray film (Kodak X-OMAT AR).

λ-Ppase treatment for cellular extract.

10 µl of a cellular extract obtained for the transfection of a 30 mm Petri-dish (0.5 x 10^6), was incubated with 4 µl (400 U/µl) λ-Ppase, buffer λ-Ppase (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij 35) and 1X $MnCl_2$. The reaction was incubated for 2 h at 30°C. The reaction was stopped with 5 µl of loading buffer.

In vivo binding assay with [^{35}S]-methionine

MA104 cells (0.5 x 10^6cells) were infected at a MOI of 3 PFU/cell of VT7/LacOI/NSP2. After 1 h of absorption, cells were transfected with 2µg of each of the NSP5 deletions mutants with 5 µl of Transfectam and induced with 1 mM IPTG. After 4 h, cells were starved in DMEM-methionine for 30 min and then the medium was replaced by DMEM with 1.5 mg/L of methionine, 1 mM IPTG, 100 µCi [^{35}S] methionine and the cells were incubated for 18 h. After incubation cells were washed 2 times in PBS and incubated 10 min in 25 mM DSP (Dithiobis(succinimidyl propionate),Pierce) at 4°C. Cells were washed 3 times in 2,5 ml 50 mM Tris-HCl pH 7.5/ 150 mM NaCl and lysed in 60 µl TNN lysis buffer(100 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5% NP-40, 1X protease inhibitor cocktail(Sigma)), incubated for 10 min in ice and centrifugated at 10,000 x g for 5 min. The supernatants were immunoprecipitated as previously described. The beads were washed twice in TNN and once in RIPA and samples were analysed by SDS-PAGE (149). Visualization of ^{35}S-labeled proteins was enhanced by fluorography using Amplify (Amersham). Autoradiography was performed at –70°C using X-ray film (Kodak X-OMAT AR).

Two hybrid

The yeast grown and two hybrid system were performed as described by Visintin et al., 1999 (283). Briefly, the plasmids were transformed into L40 yeast strain by using lithium acetate transformation protocol (91). Positive clones were selected by using auxotrophic markers for both plasmids and for lysine and histine prototropy. Histidine-positive clones and controls were lysed in liquid nitrogen and assayed for β-galactosidase activity (33).

Yeast strain

The yeast strain L40 contains lexA operator-responsive reporters chromosomally integrated: the genotype of L40 is Mata *his3*Δ200, *trp*1-901, *leu*2-3, 112*ade*2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-*lac*ZGAL4. minimal HI3 and GAL1 promoters fusede to multimerized LexA binding sites drive the expression of the HIS3 and LacZ coding sequences, respectively. The expression of HIS3 permits the growth of the transformed yeast in selective medium while the expression of LacZ, which encodes the enzyme β-galactosidase, can be monitored using a colorimetric assay based on the activity of β-galactosidase: the lacZ$^+$ yeasts form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). This strain is deficient for TRP and LEU (auxotrophic phenotype) and cannot grow on minimal medium lacking those nutrients unless functional TRP1 and LEU2 genes are introduced. Moreover, this strain carries the ade2 mutation, which confers a red colour (due to a red pigment accumulation) on medium containing limiting amounts of adenine that turns darker as the colony age.

Localization in viroplasms and quantification

Cells were transfected as described above. After 48 h of transfection with the protein fused to EGFP, cells were infected with rotavirus strains SA11 or OSU. At 5 h post-infection, cells were fixed and the immunofluorescence was performed as described. The samples were observed in a confocal microscope (Axiovert, Zeiss). The area of the viroplasms was measured with the overlay function

present in the adquisition program of the confocal and calculated in the next form:

area viroplasm={∑ [∑(area viroplasms per cell)/(number viroplasms per cell)]}/b

In which, b=number of cell

For each time point were counted the number of viroplasms of tweenty cells. The data were processed in the MS Excel software.

RESULTS

Mapping and characterisation of NSP5 phosphorylation

NSP5 activates cellular kinase(s).

The complex pattern of NSP5 phosphorylation in virus infected cells has been proposed to be due, in part, to autophosphorylation. However, no clear evidence of NSP5 enzymatic activity has been reported. In order to investigate the putative NSP5 kinase activity, we developed an *in vitro* phosphorylation assay which allowed us to obtain the characteristic PAGE mobility shift of hyper-phosphorylated NSP5 (5). We selected as a substrate in this assay the *in vitro* translated NSP5 mutant Δ1 (see figure 7 for description of all mutants) for the following reasons: i) it appears as a not hyper-phosphorylated band of ~ 20 kDa in SDS-PAGE (figure 8A, lane 1), ii) it becomes hyper-phosphorylated when expressed in MA104 transfected cells (4, 77) in a similar way to wild type NSP5 in infected cells, and iii) it is one of the most efficiently phosphorylated of all mutants tested.

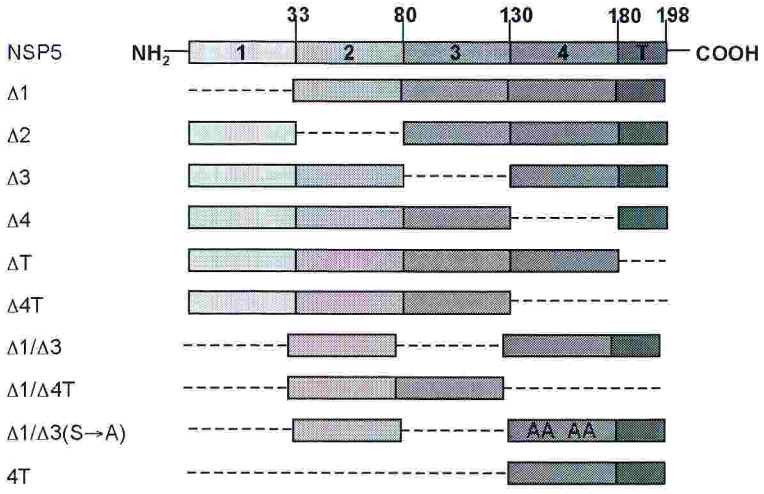


Figure 7. Schematic representation of NSP5 and mutants. Dotted lines correspond to deleted regions. A, Ser→Ala mutations.

The kinase activity, determined as the capability to instruct PAGE mobility shift of [^{35}S] labelled Δ1 protein, was investigated in cellular extracts derived from either

untransfected cells or from cells transfected with NSP5 or NSP5 deletion mutants. Extracts containing mutants Δ1, Δ3 and Δ1/Δ3, but not extracts from mock-transfected cells had phosphorylation activity (figure 8A). In agreement with this result, these three mutants are hyper-phoshorylated *in vivo* in transfected cells ((77) and figure 4). Conversely, extracts from cells transfected with wild type NSP5 showed a marginal effect, consistent with its low phosphorylation in transfected cells, in the absence of viral replication (5).

Treatment with lambda protein phosphatase (λ-Ppase) confirmed that PAGE mobility changes corresponded to hyper-phosphorylated forms of the substrate (figure 8B). Interestingly, extracts transfected with the two mutants that lack region 3 (Δ3 and Δ1/Δ3), showed the highest phosphorylation activities. Extracts from cells transfected with other mutants, such as Δ2, Δ4, ΔT or Δ1/Δ3/ΔT did not show significant activity. Table 4 reports the activities of all mutants tested. In all cases, similar amounts of transfected proteins were used, as judged by Western blot analysis. From these results it appears that domains 2, 4 and T are absolutely required for extracts' activities.

All other mutants were also tested as substrates in this assay (table 4). Only three of them, namely Δ1, Δ2 and Δ3 were positive, while all others were negative. The phosphorylated shifted forms of Δ1 were only achieved with extracts from cells transfected with NSP5 mutants that showed hyper-phosphorylation *in vivo*. This result suggested that, either the activity resides in the transfected protein itself or, alternatively, the transfected protein induces or activates an otherwise inactive cellular kinase(s).

To discriminate between these two hypothesis, a His_6 tagged version of Δ1/Δ3 protein was purified from transfected cellular extracts on a nickel column. The purified protein was analysed by Western immunoblotting (figure 8C). As shown in figure 8D, the cellular extract containing His_6-Δ1/Δ3 showed phosphorylation activity (lane 5), while the same amount of the purified protein did not (lane 7).

The possibility that the transfected NSP5 mutant is complexed with a cellular kinase, thus activating phosphorylation of NSP5 itself seems unlikely, since addition of purified His_6-Δ1/Δ3 to a mock cellular extract did not reconstitute the

activity (lane 6). This suggests that expression of the protein is necessary to promote activation of the cellular kinase(s). Hyper-phosphorylation of NSP5 in virus infected cells occurs also in the presence of Actinomycin D indicating that transcription of cellular genes are not required for the emergence of the kinase(s) activity (Figure 8E). Phosphorylation and capacity to activate the cellular kinase(s) appear to be distinct characteristics of NSP5. Indeed, mutation of four serine residues within domain 4 [mutant Δ1/Δ3(S→A)], that completely abolished phosphorylation of the protein expressed *in vivo* (see below), did not affect its ability to activate the cellular kinase(s) (figure 8D).

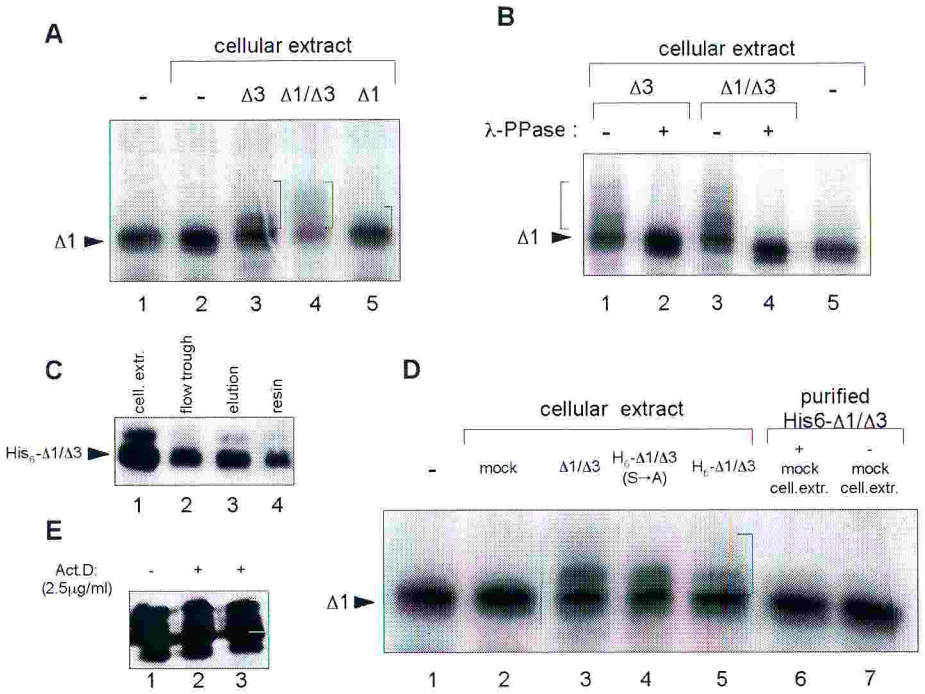


Figure 8. *In vitro* phosphorylation assay. Analysis of immunoprecipitates of *in vitro* translated, [^{35}S]-methionine labelled mutant Δ1. A, substrate was incubated with cellular extracts from cells transfected with the indicated mutants; mock indicates extracts from cells transfected with the same plasmid without insert. B, λ-Ppase treatment as indicated. C, Purification of His_6-Δ1/Δ3 by nickel column, the different steps of purification are indicated. The samples were visualized with a western immunoblotting using an anti-NSP5 serum. D, purified His_6-Δ1/Δ3 was obtained by nickel column purification and the same amount of lane 5 used in lanes 6 and 7. Arrowheads indicate the unphosphorylated Δ1 substrate and vertical brackets the position of mobility shifted phosphorylated forms. E, Treatment of cells infected with rotavirus SA11 (4 hours) with actinomycin D (2,5μg/ml). Lane 2, treatment with actinomycin D for 4 hours post-infection and lane 3, treatment of cells 1.5 hours pre-infection and 4 hours post-infection. Samples were visualized by western immunoblotting with an anti-NSP5 serum. NSP5 hyper-phosphorylation is indicated by bracket.

Mapping phosphorylation sites of NSP5.

In order to map the phosphorylation sites on NSP5, we constructed a variety of NSP5 deletion mutants, as GST fusion proteins. None of these proteins produced in bacteria showed any phosphorylation activity (figure 9), neither they were phosphorylated by His_6-Δ1/Δ3 purified from transfected cells. These evidences strengthen the idea that NSP5 has not kinase activity. However, some of the mutants, as well as wild type NSP5, served as substrates when incubated *in vitro* with a cellular extract from Δ1/Δ3 transfected cells, as a source of enzyme. As shown in figure 9 only mutants containing region 4 were phosphorylated indicating that most of the phosphorylation occurs within this domain, and suggesting that *in vivo* phosphorylation sites could reside in this region. A similar category of experiment, was carried out with NSP5 deletion mutants expressed in transfected mammalian cells, and then phosphorylated *in vitro* in total cellular extracts. For this, the cellular extracts were incubated with [^{32}P]-γ-ATP and immunoprecipitated with an anti-NSP5 antibody. Figure 10, shows that the region 4 is required but, also the tail (T) is necessary for this incorporation (figure 10A lane 1 and 2). On the other hand, hyper-phosphorylation was observed in mutants Δ3 and Δ1/Δ3, while the only presence of regions 4 and T (lane 8) was not enough to produce the typical mobility shift. This suggests that in this process region 2 must be involved.

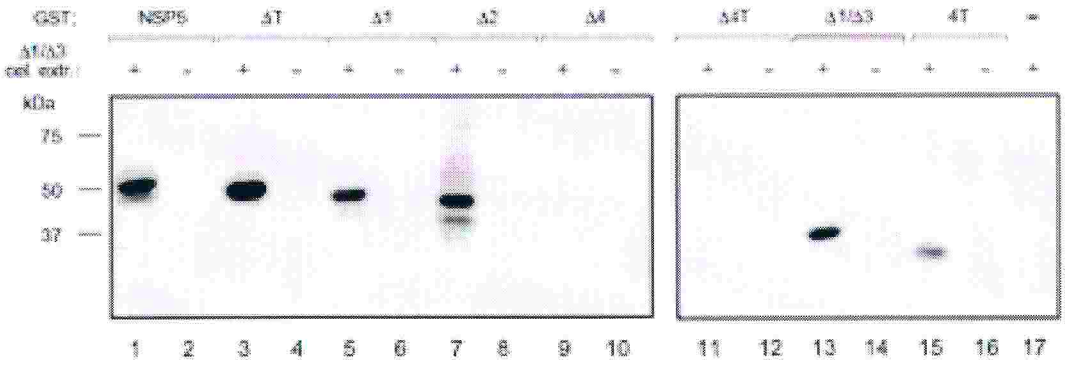


Figure 9. Mapping phosphorylation sites of NSP5. SDS-PAGE analysis of immunoprecipitated, *in vitro* phosphorylated GST-NSP5 mutant proteins (0.2 μg) incubated with and without cellular extract from Δ1/Δ3 transfected cells. GST protein negative control (lane 17) was loaded without immunoprecipitating.

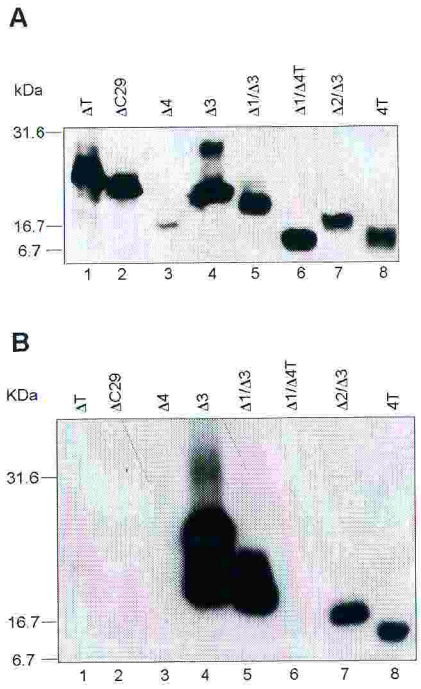


Figure 10: *In vitro* [^{32}P]-γ-ATP incorporation of cellular extracts transfected with NSP5 mutants. A, Western immunoblotting of each independently transfected construct. B, Immunoprecipitation of cellular extracts transfected with the indicated mutant constructs and labelled *in vitro* with [^{32}P]-γ-ATP. Relative molecular masses in kDa are shown to the left.

We have previously reported that NSP5 phosphorylation takes place in Ser and Thr residues (5). To further characterise phosphorylation sites we mutated to alanine (in mutant Δ1/Δ3) four serines (Ser^{153}, Ser^{155}, Ser^{163} and Ser^{165}) within region 4, present in a particular acidic amino acid context (AD**S**D**S**EDYVLDD**S**D**S**DDG) (figure 11A) and tested them for their ability to be phosphorylated *in vivo*. Figure 11B shows immunoprecipitates of several transfected Δ1/Δ3 Ser→Ala mutants following labelling with [$^{32}P_i$]. The four serines appeared to be sites of phosphorylation. The only mutant that showed no phosphorylation was the one with all four serines mutated (lane 9). All lanes in figure 11B contained comparable amounts of transfected protein, determined by Western immunoblotting (not shown). The double band corresponded to low mobility hyper-phosphorylated forms as judged by λ-Ppase sensitivity (figure 11C). Further confirmation that the four serines within region 4 are the main phosphorylation sites was obtained with a GST-Δ1/Δ3 fusion protein with all four mutated serines [GST-Δ1/Δ3(S→A)] which was not phosphorylated by Δ1/Δ3 cellular extracts (figure 11D).

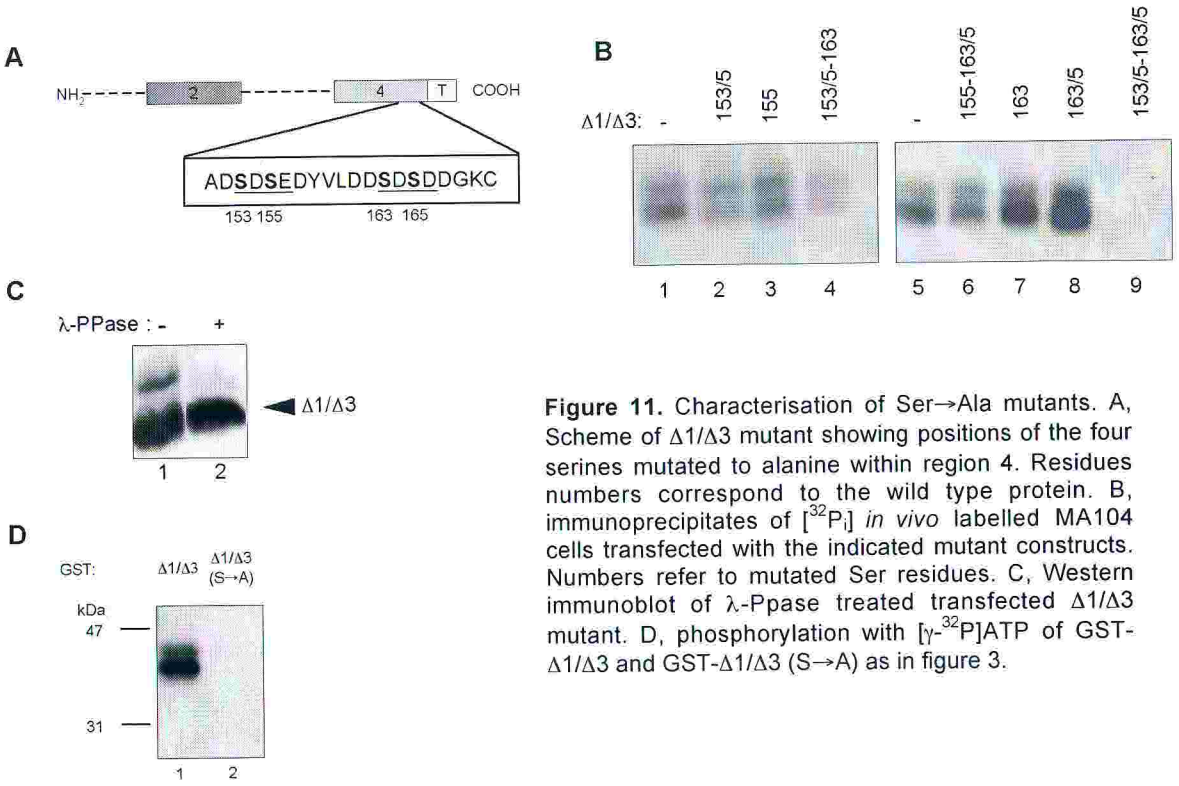


Figure 11. Characterisation of Ser→Ala mutants. A, Scheme of Δ1/Δ3 mutant showing positions of the four serines mutated to alanine within region 4. Residues numbers correspond to the wild type protein. B, immunoprecipitates of [$^{32}P_i$] *in vivo* labelled MA104 cells transfected with the indicated mutant constructs. Numbers refer to mutated Ser residues. C, Western immunoblot of λ-Ppase treated transfected Δ1/Δ3 mutant. D, phosphorylation with [γ-^{32}P]ATP of GST-Δ1/Δ3 and GST-Δ1/Δ3 (S→A) as in figure 3.

NSP5 is substrate of CK2.

The sequence of NSP5 region 4 containing the four phosphorylated serines, **S**D**S**E and **S**D**S**D are characteristic of substrates of casein kinase II (CK2) ((171), Prosite-EMBL/http://www.ebi.ac.uk). We therefore tested whether CK2 was able to phosphorylate the different GST fusions. As shown in figure 12A, CK2 was able to phosphorylate NSP5 and all mutants containing region 4, namely Δ1/ΔT, Δ1/Δ3 and Δ2. This phosphorylation appeared to be restricted to serines 153, 155, 163 and 165, since mutant GST-Δ1/Δ3(S→A) was not phosphorylated by CK2. This result is in complete agreement with the lack of phosphorylation of this mutant *in vivo* (figure 12B). CK2 was also able to phosphorylate the *in vitro* translated, His_6-Δ1 substrate, as demonstrated by the results shown in figure 12B. The left panel shows both, the [^{35}S] label of the *in vitro* translated substrate and the [^{32}P] label from [γ^{32}P] ATP. On the right panel only the [^{32}P] radioactivity was detected. In these conditions CK2 was able to convert, similarly to the

transfected cellular extracts, part of the substrate into mobility shifted forms. Phosphorylation with CK2 of the GST-fusions was also performed in the conditions used with the cellular extracts, with similar results. These results suggested that a CK2-like enzymatic activity is involved in NSP5 phosphorylation.

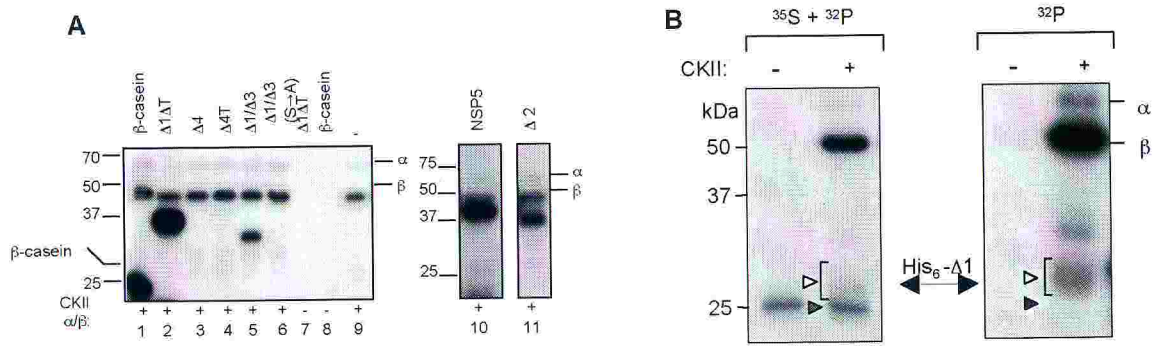


Figure 12. NSP5 is a substrate of casein kinase II. SDS-PAGE analysis of an *in vitro* CK2 phosphorylation assay with [γ^{32}P]ATP. A, the indicated GST-NSP5 mutants were used as substrates. Positive and negative controls are indicated in lanes 1, 7, 8 and 9. B, purified, *in vitro* translated [^{35}S]His$_6$-Δ1 was used as substrate in the presence or absence of CK2. The two panels show autoradiography of the same gel, detecting ^{35}S+^{32}P (left) and ^{32}P (right), respectively. Filled and open arrowheads indicate His$_6$-Δ1 precursor and hyper-phosphorylated forms, respectively. Autophosphorylated GST-α and GST-β CK2 subunits are indicated.

Localization to viroplasms

NSP5 and NSP2 are the two rotavirus non-structural proteins that localise in viroplasms in the cytoplasm of infected cells. We have previously shown that the two transfected proteins directly interact *in vivo* producing VLS (77). In order to investigate the requirements of NSP5 to localise in true viroplasms in infected cells, we analysed the fate of several NSP5 mutants fused to EGFP. Cells were transfected with different constructs, infected with rotavirus (SA11 strain) 48 hrs later, and fixed at 6 hrs post-infection. Immunofluorescence was performed on a confocal microscope and viroplasms revealed with anti-NSP2 serum.

Figure 13 shows that, in non-infected cells, NSP5 wild type and mutants have a diffused distribution in the cytoplasm. Following infection this distribution was highly affected both for NSP5 and for some of the deletion mutants, such as Δ1,

Δ2, Δ3 and Δ1/Δ3, which became rapidly re-localised in viroplasms. On the contrary, mutants lacking region 4 (such as Δ4) or the carboxy-terminal tail (ΔT) (not shown) did not localise to viroplasms. Table 4 summarises the results obtained with all mutants tested. Interestingly, mutant Δ1/Δ3(S→A), with all four serines in region 4 mutated, that we previously demonstrated not to be phosphorylated *in vivo* (see above), showed a clear co-localisation in viroplasms. This result suggested that, although the presence of the carboxy-terminal regions 4 and T are required, phosphorylation is not essential for NSP5 localisation to viroplasms, in the context of infected cells.

An interesting observation regards mutant Δ2 which does not get phosphorylated *in vivo* and does not form VLS (77), whereas it localised to viroplasms (Fig 13). This is most likely due to interaction with wild type NSP5 that was shown to depend on the last ten carboxy-terminal amino acids (276). This interpretation is supported by the experiment shown in figure 14. Localisation to VLS was only obtained when Δ2-EGFP was co-transfected with NSP2 into a cell line stable expressing wtNSP5 (MA104-C7) (5), and not when co-transfected into a cell not expressing it.

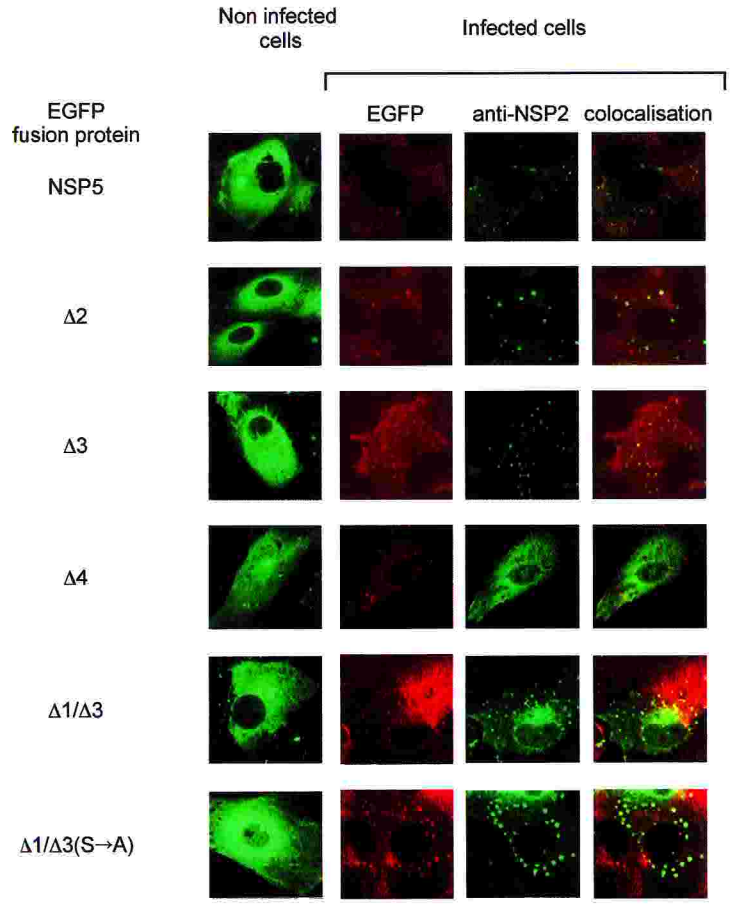


Figure 13. Confocal immunofluorescence. MA104 cells were transfected with NSP5 mutants fused to EGFP followed by infection with rotavirus. Viroplasms were detected with an anti-NSP2 (red) antibody. The rightmost column is the superimposition of the two independently acquired images.

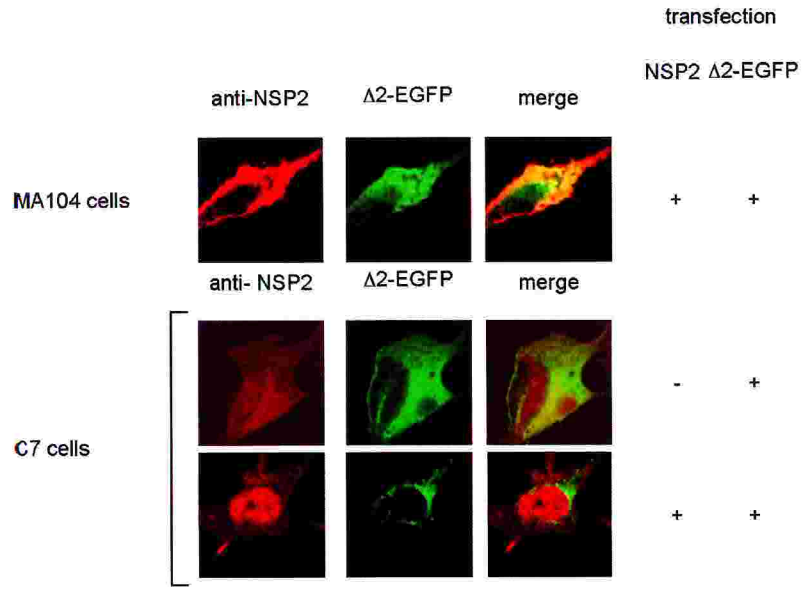


Figure 14. Formation of VLS (viroplasms like structure) in C7 cells. C7 cells (MA104 expressing NSP5) were transfected with Δ2-EGFP and NSP2. When NSP2 is co-transfected the formation of VLS can be visualised. This suggest that for interaction with Δ2 -EGFP is necessary the presence of NSP5 (C7 cells) and NSP2.

Table 4. Summary of NSP5 properties

protein	Presence of property[a]		
	Localisation to viroplasms	Kinase activity of cellular extract[b]	Activity as substrate[c]
NSP5	+	-	±
Δ1	+	+	+
Δ2	+	-	+
Δ3	+	+	+
Δ4	-	-	-
ΔT	-	-	-
Δ1/Δ4T	-	-	-
Δ4T	-	ND	-
4T	+	-	-
Δ1/Δ3	+	+	-
Δ1/Δ3(S→A)	+	+	-
Δ1/Δ3/ΔT	ND	-	-

[a] +,present;-, absent;±, marginal; ND, not determined.
[b] *In vitro* translation/ phosphorylation assay with NSP5-Δ1 used as substrate.
[c] *In vitro* translation/phosphorylation assay. Δ1/Δ3 cellular extract used as a source of cellular kinase(s).

In vivo hyper-phosphorylation assay

All together, the results presented indicate that the hyper-phosphorylation of NSP5 is an autoregulated process, which makes it to appears as an autophosphorylation. In addition, it is also apparent that the kinase activity can be detected in cells extracts transfected with some, but not all, NSP5 deletion mutants. We therefore hypothesised the existence in NSP5 of regions specific for the activation of a cellular kinase and its own phosphorylation. To test this hypothesis *in vivo*, we developed a transfection assay, where two mutants of NSP5 were chosen as substrate. One mutant Δ2, was used since it is not phosphorylated *in vivo* (77), while it is a good substrate *in vitro* (see table 4). The second one mutant Δ4, was used since it is not phosphorylated *in vivo* (77) and is not a substrate *in vitro* (see table 4). To distinguish these two mutants from other mutants used as activators of cellular kinases in a co-transfection assay, the eleven amino acids SV5 tag was added at the N-terminus of the substrate proteins(figure 15A). A co-transfection assay was performed using as activators

Δ1/Δ3 and Δ3 with the substrates SV5-Δ2 or SV5-Δ4. To visualize the effect of the activators with the different substrates, the cellular extracts were loaded in a SDS-PAGE gel and a Western blot anti-SV5 was performed. The results presented in figure 15 show that the SV5-Δ2 substrate is able to produce a mobility shift in the presence of the kinase activators (figure15B, lanes 2 and 3). In contrast, the SV5-Δ4 substrate was not able to produce this mobility shift (lanes 5 and 6). A treatment with λ-Ppase confirmed that the mobility shift present in these samples corresponded to hyper-phosphorylation of the SV5-Δ2 substrate (figure 15C). These results confirmed that the hyper-phosphorylation sites are present in the region 4 of NSP5. Similarly to what was observed in the *in vitro* translation/phosphorylation assay (figure 8A and D), Δ1/Δ3 and Δ3 were also able to induce hyper-phosphorylation of the SV5-Δ2 substrate *in vivo*.

As described above, the minimal protein necessary for the activation of the cellular kinase in the *in vitro* translation/phosphorylation assay contains regions 2, 4 and T. In order to discriminate the role of each NSP5 region in the activation of the cellular kinase, we co-expressed SV5-Δ2 with the NSP5 deletion mutants Δ1, Δ2, Δ3 and ΔT. As shown in figure 15, only mutants Δ1 and Δ3 were able to hyper-phosphorylate SV5-Δ2, demonstrating that also *in vivo*, regions 2, 4 and T (present in mutant Δ1/Δ3) are required. These results are consistent with the inhibitory effect caused for the regions 1 and 3 (table 4), in absence of one or both of these regions, the NSP5 mutants are able to be activator of cellular kinase. For this reason, NSP5, Δ2, Δ4 and ΔT are not activators *in vivo*.

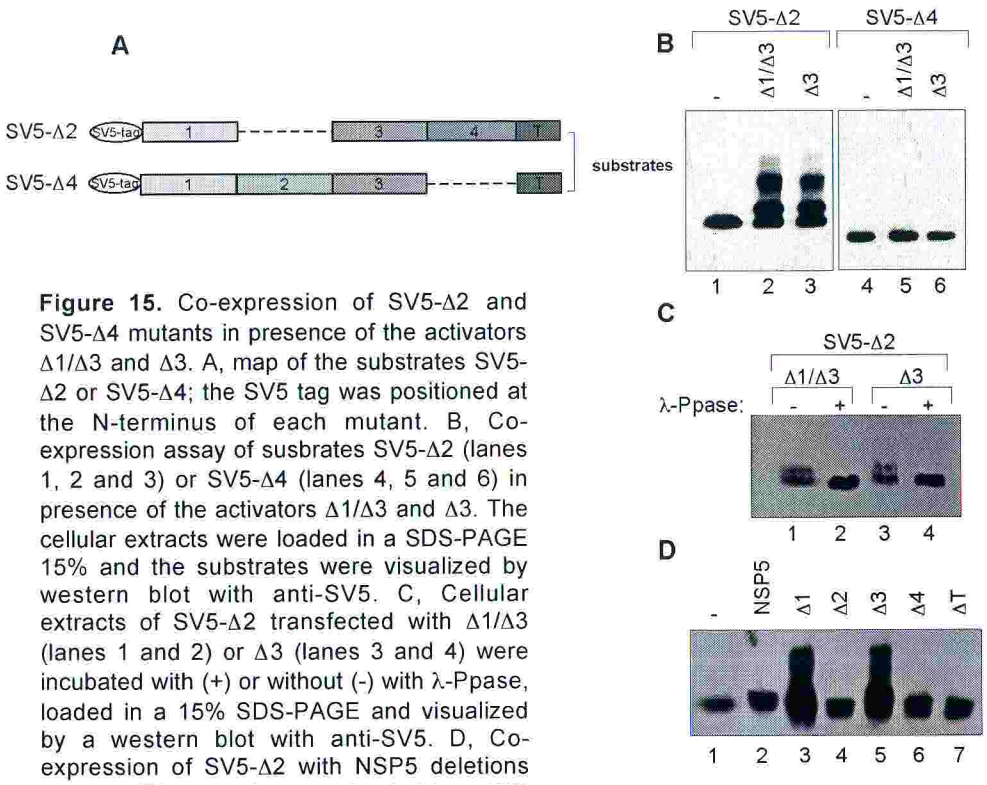


Figure 15. Co-expression of SV5-Δ2 and SV5-Δ4 mutants in presence of the activators Δ1/Δ3 and Δ3. A, map of the substrates SV5-Δ2 or SV5-Δ4; the SV5 tag was positioned at the N-terminus of each mutant. B, Co-expression assay of susbrates SV5-Δ2 (lanes 1, 2 and 3) or SV5-Δ4 (lanes 4, 5 and 6) in presence of the activators Δ1/Δ3 and Δ3. The cellular extracts were loaded in a SDS-PAGE 15% and the substrates were visualized by western blot with anti-SV5. C, Cellular extracts of SV5-Δ2 transfected with Δ1/Δ3 (lanes 1 and 2) or Δ3 (lanes 3 and 4) were incubated with (+) or without (-) with λ-Ppase, loaded in a 15% SDS-PAGE and visualized by a western blot with anti-SV5. D, Co-expression of SV5-Δ2 with NSP5 deletions mutants. The samples were loaded in a 15% SDS-PAGE and visualized by Western immunoblotting with an anti-SV5.

Characterisation of motifs *a*, *b* and *c* of NSP5.

As described above, SV5-Δ2 was a good substrate both in the *in vivo* hyper-phosphorylation assay as well as in the *in vitro* translation/phosphorylation kinase assay. On the other hand, Δ2 was unable to induce its own phosphorylation. Thus, we decided to study role of the region 2 in the activation of the putative cellular kinase, based also in the following evidences: i) as described above the main regions important for activation of the cellular kinase are the regions 2, 4 and T; ii) however, the presence of the four serines (153, 155, 163 and 165) of region 4 appear to have a role in the basal phosphorylation of NSP5 rather than in the activation process (figure 11A) and iii) there is activation of the cellular kinase even when these four serines are mutated to alanines (figure 8), thus suggesting that it is the region 2 the candidate to play a role in the activation of the kinase involved in the NSP5 hyper-phosphorylation.

A sequence analysis of region 2 shows the presence of two interesting motifs, that we named motif *a* and *b*. Motifs *a* and *b* contain the sequence SDSAS and SFSIRS, respectively. However, these motifs were not identified as substrates of any cellular kinase by computing analysis. We then decided to investigate if point mutations of serines to alanines in either motif *a*, *b* or both had any effect in the ability of region 2 to activate the cellular kinase. Mutant Δ3 was chosen to introduce these mutations generating mutants Δ3a (with all three serines of motif *a* mutated to alanines, Δ3b (the three serines of motif *b* mutated to alanines) and Δ3ab (both motifs *a* and *b* mutated). The four serines (153, 155, 163 and 165) present in region 4 will be referred as motif *c*. A map of these new mutants is presented in figure 16A. We first tested the *in vitro* activity of the cellular extracts transfected with these new constructs to phosphorylated them, by incubating with [^{32}P]-γ-ATP while mutant Δ3b was as active as Δ3, mutants Δ3a and Δ3ab were not able to be phosphorylated *in vitro* (Figure 16B). Moreover, the same cellular extract (Δ3a) is not active in the *in vitro* translation/phosphorylation kinase assay, because is not able to hyper-phosphorylate the [^{35}S]-Δ1 substrate (Figure 16C, lane 5). On the other hand, mutations in motif *b* (Δ3b) did not affect activity of the cellular extract (figure 16C, lane 6). More importantly, *in vivo* co-expression of substrate SV5-Δ2 with Δ3a, Δ3b or Δ3c as activators (Figure 16D, lanes 2, 3 and 4) showed that only mutations in motif *a* abolish the capacity to hyper-phosphorylate SV5-Δ2. Taken together, these results indicate that serines present in motif *a* play a crucial role in the activation of the cellular kinase involved in NSP5 hyper-phosphorylation.

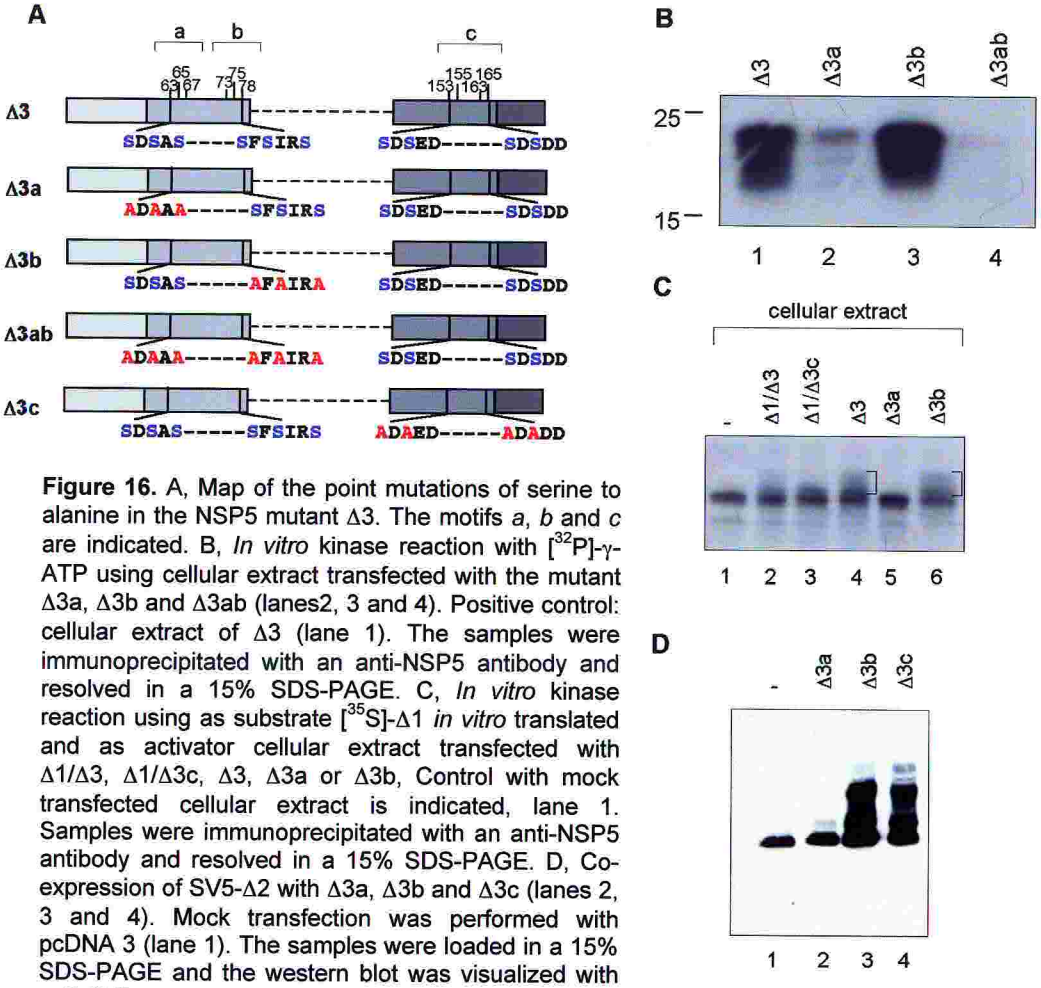


Figure 16. A, Map of the point mutations of serine to alanine in the NSP5 mutant Δ3. The motifs *a*, *b* and *c* are indicated. B, *In vitro* kinase reaction with [^{32}P]-γ-ATP using cellular extract transfected with the mutant Δ3a, Δ3b and Δ3ab (lanes2, 3 and 4). Positive control: cellular extract of Δ3 (lane 1). The samples were immunoprecipitated with an anti-NSP5 antibody and resolved in a 15% SDS-PAGE. C, *In vitro* kinase reaction using as substrate [^{35}S]-Δ1 *in vitro* translated and as activator cellular extract transfected with Δ1/Δ3, Δ1/Δ3c, Δ3, Δ3a or Δ3b, Control with mock transfected cellular extract is indicated, lane 1. Samples were immunoprecipitated with an anti-NSP5 antibody and resolved in a 15% SDS-PAGE. D, Co-expression of SV5-Δ2 with Δ3a, Δ3b and Δ3c (lanes 2, 3 and 4). Mock transfection was performed with pcDNA 3 (lane 1). The samples were loaded in a 15% SDS-PAGE and the western blot was visualized with anti-SV5.

Characterisation of serines from motif *a*

With the purpose to further analyse which of the three serines of motif *a* is involved in regulating the activation of the cellular kinase, we point mutated one by one the three serines, creating mutants Δ3/S63A, Δ3/S65A and Δ3/S67A. A map of the three serines is presented in figure 17A. A kinase reaction using cellular extracts transfected with these mutants was performed in the presence of [^{32}P]-γ-ATP and [^{32}P]-γ-GTP (Figure 17B). Samples were phosphorylated by both nucleotides (ATP and GTP) indistinguishable. Nevertheless, the mutant in serine 67 seems not to be phosphorylated in these conditions. Studies of these cellular extracts as activators were also performed with the *in vitro*

translation/phosphorylation assay. For this, [^{35}S]-Δ1 protein was used as substrate of the kinase assay and incubated with the mutants. The results obtained showed that the cellular extract Δ3/S67A was not able to hyper-phosphorylate [^{35}S]-Δ1 (Figure 17C). These results indicated that serine in position 67 appears to have a regulatory role in the activation of the cellular kinase.

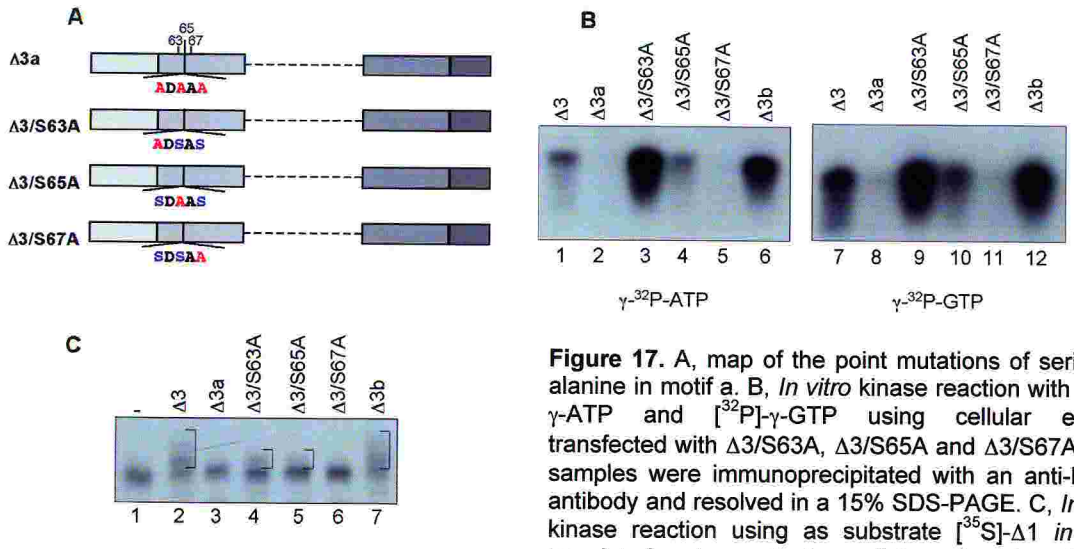


Figure 17. A, map of the point mutations of serine to alanine in motif a. B, *In vitro* kinase reaction with [^{32}P]-γ-ATP and [^{32}P]-γ-GTP using cellular extract transfected with Δ3/S63A, Δ3/S65A and Δ3/S67A. The samples were immunoprecipitated with an anti-NSP5 antibody and resolved in a 15% SDS-PAGE. C, *In vitro* kinase reaction using as substrate [^{35}S]-Δ1 *in vitro* translated and as activator, cellular extract transfected with Δ3/S63A, Δ3/S65A and Δ3/S67A (lanes 4, 5 and 6).Controls are indicated (lanes 1, 2, 3 and 7). The samples were immunoprecipitated with an anti-NSP5 antibody and resolved in as 15% SDS-PAGE.

Due to the importance attributed to serine 67 in the process of activation, we decide to study if this serine in particular must be phosphorylated in order to permit the hyper-phosphorylation of the full protein. For this purpose, we made a new construct were serine 67 was substituted by an aspartic acid, Δ3(S63,65A/S67D) while the two other serines of motif *a* serines 63 and 65 were mutated to alanine to avoid any possible phosphorylation of those two residues (figure18A). The aspartic acid substitution mimics the negative charge of a phosphate group, behaving as a phosphorylated serine (42, 123). As shown in figure 18B (lanes 1 and 3) mutant Δ3(S63,65A/S67D) was able to be phosphorylated similarly to the non-mutated Δ3 in the presence of [^{32}P]-γ-ATP. This result indicates that the presence of the aspartic acid in position 67 mimics a

phosphorylated serine, allowing the hyper-phosphorylation of the full protein. This was indeed confirmed *in vivo*, by co-expression with the substrate SV5-Δ2. The results obtained clearly show that mutant Δ3(S63,65A/S67D) activators hyper-phosphorylation of the Δ2 substrate in the same form as Δ3 (Figure 18C, Lanes 2 and 5). On the other hand, mutants which, have the S67A mutation were not able to hyper-phosphorylate SV5-Δ2.

These results demonstrate that serine 67 of Δ3-NSP5 is phosphorylated *in vivo* and this phosphorylation is necessary for the activation of a cellular kinase, which in turns determines the hyper-phosphorylation. However, it remains to be identified which is the cellular kinase involved in the phosphorylation of the serine 67 as well as the one activated by a region 2 phosphorylated in serine 67. Interestingly, the sequence present in motif *a* could be part of a new substrate sequence for a cellular kinase.

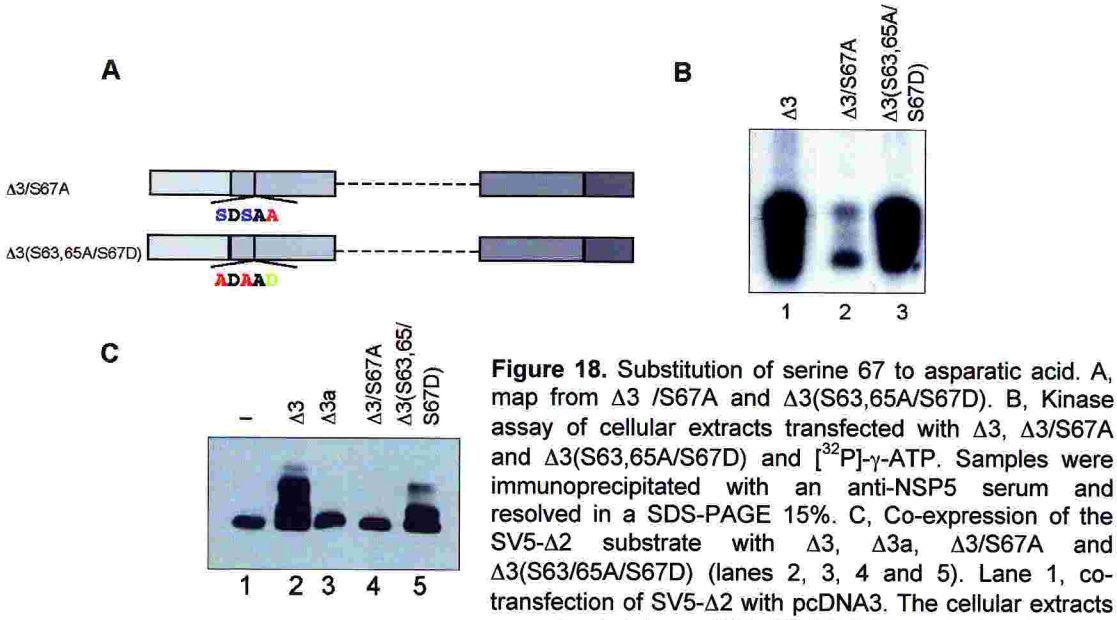


Figure 18. Substitution of serine 67 to asparatic acid. A, map from Δ3 /S67A and Δ3(S63,65A/S67D). B, Kinase assay of cellular extracts transfected with Δ3, Δ3/S67A and Δ3(S63,65A/S67D) and [^{32}P]-γ-ATP. Samples were immunoprecipitated with an anti-NSP5 serum and resolved in a SDS-PAGE 15%. C, Co-expression of the SV5-Δ2 substrate with Δ3, Δ3a, Δ3/S67A and Δ3(S63/65A/S67D) (lanes 2, 3, 4 and 5). Lane 1, co-transfection of SV5-Δ2 with pcDNA3. The cellular extracts were loaded in a 15% SDS-PAGE and visualized by Western blot anti-SV5.

Role of region tail (T) in the activation of the cellular kinase.

The crucial role of the tail was demonstrated by co-expressing activators Δ1/Δ3 or Δ1 that lack the tail, with substrate SV5-Δ2 (figure 19A). As shown seen in figure 19C, while both Δ1 and Δ1/Δ3 activate the cellular kinase, the two tail-less version do not. This result suggest that either the tail (T) is also important for the activation of the cellular kinase, or, alternatively, it plays a role as a dimerising domain, as reported by Torres-Vega *et al.* , 2000 (276), allowing the phosphorylation *in trans* by a kinase activated by another NSP5 molecule containing domain 2. An alternative construct to be used as substrate is needed, in which the tail is deleted. Such construct SV5-Δ2/ΔT is at present being constructed. The results will be presented at the moment of the thesis presentation.

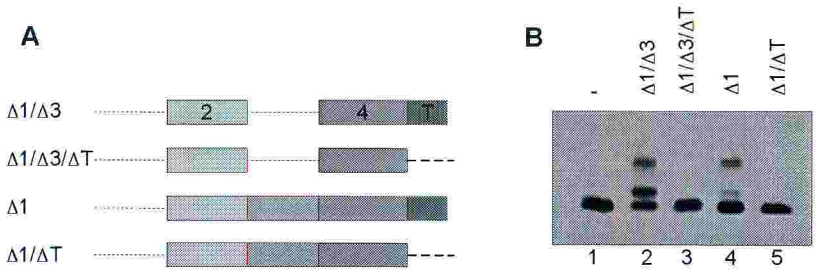


Figure 19. Role of tail in the kinase activation. A, Map of the activators used in the assays. B, Co-expression of the substrate SV5-Δ2 with Δ1/Δ3 and Δ1 with and without tail. The samples were loaded in a 15% SDS-PAGE and visualized by Western immunoblotting with an anti-SV5.

Motif *a* mutations in full-length NSP5

The results presented through this thesis have shown that the serines present in motif *a* (SDSAS) have an important role in the activation a cellular kinase, which would the hyper-phosphorylate the full NSP5 protein. It has been demonstrated that serine 67 needs to be phosphorylated to trigger the hyper-phosphorylation process. Since these conclusion were obtained with deletions mutants (mainly Δ3) we wanted to investigated the role of these serines in the full-length NSP5 protein. In order to do this, three mutants of NSP5, were constructed. Containing mutation in motif *a* namely NSP5(S63,65,67A), NSP5/S67A and

NSP5(S63,65A/S67D). A scheme of the NSP5 mutant is presented in figure 20A. First, we studied the activation function of these mutants in the *in vivo* co-expression experiment, using SV5-Δ2 as substrate. The results of this experiment showed that NSP5(S63,65A/S67D) was the mutant with highest ability to hyper-phosphorylate the substrate (figure 20B). Mutant NSP5/S67A showed partial activity probably due to the fact that two serines (in position 63 and 65) are still present indicating that the relevant role of serine 67 in the context of the wt NSP5 is less stringent than in Δ3. In order to assess the degree of phosphorylation of the NSP5 mutants we also performed a $^{32}P_i$ *in vivo* labelling and immunoprecipitation of transfected cells. Equal amounts of protein were used for this assay, as determined by western immunoblotting (not shown). As seen in the figure 20C, all the mutants displayed a comparable basal phosphorylation (open arrowheads), with the only exception of the mutant NSP5(S63,65A/S67D), which has an additional band (solid arrowhead) that corresponds to a hyper-phosphorylated form. The hyper-phosphorylation obtained with this NSP5(S63,65A/S67D) mutant resemble the one obtained when NSP5 was co-transfected with NSP2 (figure 3, introduction) (4), suggesting that phosphorylation of serine 67 (mimicked by an aspartic acid in this position) may be the consequence of the interaction with NSP2.

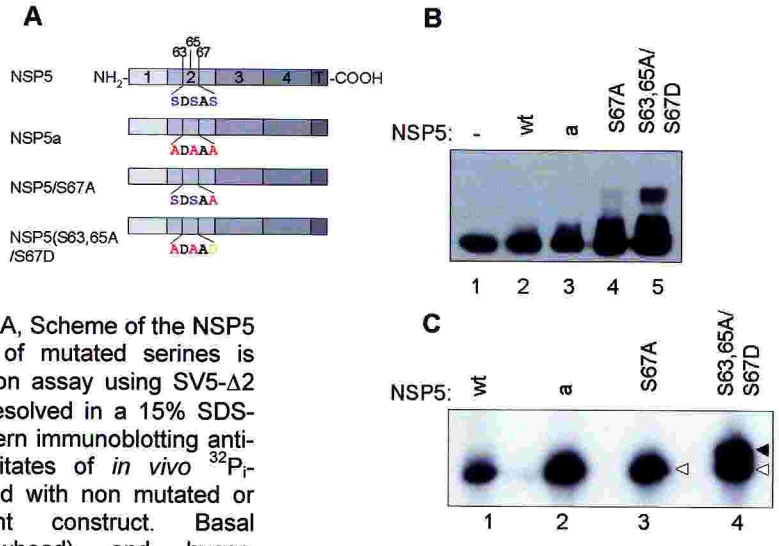


Figure 20. NSP5 point mutants. A, Scheme of the NSP5 point mutations. The position of mutated serines is indicated. B, In vivo co-expresion assay using SV5-Δ2 substrate. The samples were resolved in a 15% SDS-PAGE and visualized in a Western immunoblotting anti-SV5 serum. C, Immunoprecipitates of *in vivo* $^{32}P_i$-labelled MA104 cells transfected with non mutated or with the indicated mutant construct. Basal phosphorylation (open arrowhead) and hyper-phosphorylation (solid arrowhead) are indicated.

Interaction of NSP5 mutants with NSP2.

As mentioned, NSP5 interacts with NSP2 (see introduction). This interaction triggers the hyper-phosphorylation of NSP5 (4) and the formation of VLS (77), when the two proteins are co-expressed in the absence of any other rotaviral protein. To investigate if this is also true when serines of NSP5 are mutated, we have co-expressed NSP5 and its mutants in the presence of NSP2, followed by Western immunoblotting with anti-NSP5 and anti-NSP2. The results presented in figure 21 indicate that, as show above, the presence of an aspartic acid in position 67 is enough to hyper-phosphorylate NSP5, even in the absence of NSP2. The presence of alanine in position 63, 65 and 67 did not have any influence in the migration of NSP5a (Figure 21A). However, the VLS formation with the NSP5 mutants was not affected, even though these proteins were not hyper-phosphorylated (Figure 21B). Taken together, these results strongly suggest that the hyper-phosphorylation is not required for VLS formation. Nevertheless, it cannot be ruled out that a basal phosphorylation is necessary for formation of these structures.

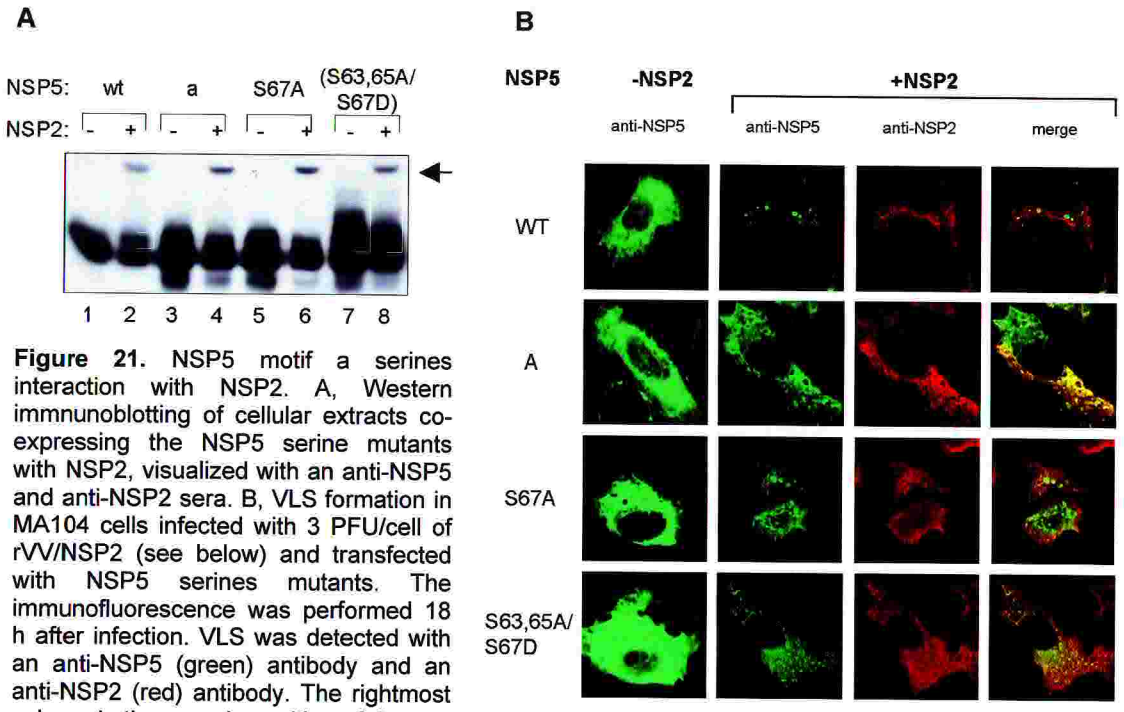


Figure 21. NSP5 motif a serines interaction with NSP2. A, Western immnunoblotting of cellular extracts co-expressing the NSP5 serine mutants with NSP2, visualized with an anti-NSP5 and anti-NSP2 sera. B, VLS formation in MA104 cells infected with 3 PFU/cell of rVV/NSP2 (see below) and transfected with NSP5 serines mutants. The immunofluorescence was performed 18 h after infection. VLS was detected with an anti-NSP5 (green) antibody and an anti-NSP2 (red) antibody. The rightmost column is the superimposition of the two indepently acquired images.

Requirements to be substrate

All the studies showed above allowed us characterise the requirements of NSP5 to be an activator. However, the conditions to be a substrate are not well established yet. We have found that SV5-Δ2 is a good substrate *in vivo* for the hyper-phosphorylation (Figure 15), while SV5-Δ4 is not, thus suggesting that the serines necessary for the hyper-phosphorylation are present in the region 4. As described above, data obtained with GST-NSP5 fusions indicated that serines 153, 155, 163 and 165 (motif *c*) were the main phosphorylation sites. With the purpose to verify whether these serines are the key residues *in vivo*, we mutated all four of them to alanine in mutant Δ2, generating an SV5-Δ2c substrate (Figure 22A). Co-expression of this mutant with the activators Δ1/Δ3 or Δ3 showed that the substrate continues to be hyper-phosphorylated, even though the hyper-phosphorylation is less intense than the wild type substrate SV5-Δ2 (figure22B). These results indicate that other serines motif *c* are likely to participate in with the hyper-phosphorylation of NSP5.

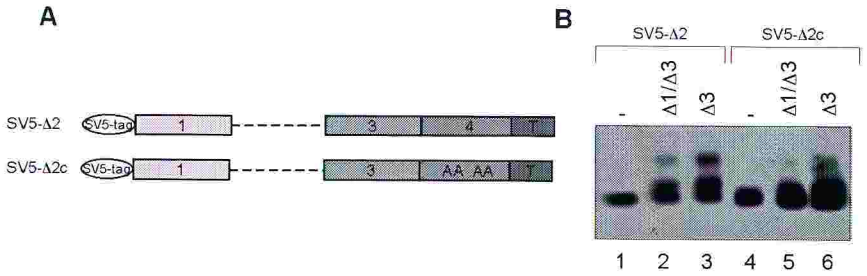


Figure 22. Requirement to be substrate. A, Scheme of SV5-Δ2c substrate. B, Western immunoblotting of cellular extracts co-expressing SV5-Δ2 or SV5-Δ2c with activators Δ1/Δ3 and Δ3. The western immnublotting was visualized with an anti-SV5 antibody.

On the other hand, mutants Δ3a, Δ3b and Δ3c were also tested as substrates in the *in vitro* translated/phosphorylation assay with Δ1/Δ3 or Δ3 cellular extracts as source of kinase. The results of this experiment presented in figure 23A, show that Δ3a and Δ3b can be hyper-phosphoryalted in agreement with all our previous finding. Δ3c, however although its shows mobility shift , it is clearly less hyper-phosphorylated than the other two substrates in similar conditions. As

demonstrated in the column plot (figure 23B), Δ3c hyper-phosphorylation is severely impaired. This result supports the idea that the hyper-phosphorylation is partially due to serines in motif *c*.

The role of two other serines in region 4 (137 and 142) is at part being investigated.

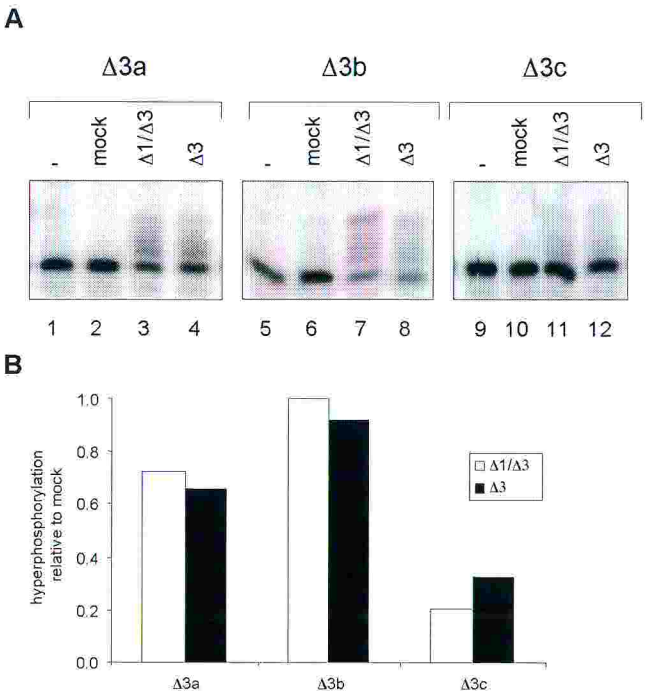


Figure 23. Δ3 motif a, b and c substrates. A, *In vitro* kinase assay using, in *vitro* [^{35}S] translations of Δ3a, Δ3b and Δ3c as substrates. The activators were cellular extracts transfected with Δ1/Δ3 and Δ3. The controls are represented with kinase assay in TNN buffer (-) and with cellular extract mock transfected (mock). B, The [^{35}S] intensity of the bands was counted in cyclone densitometer. The Δ3 mutant substrates were plotted with the ratio of hyper-phoshorylation for each activators and its controls.

Δ3a is also a substrate

As previously shown mutant Δ3a is not hyper-phosphorylated when expressed alone because of the impaired activation of cellular kinase *in vivo* (figure 16), but it can be hyper-phosphorylated when used as substrate in the *in vitro* translation/phosphorylaltion assay (figure 23). This suggests, that indeed, it could be a substrate provided that the cellular kinase is activated. To observe the behaviour of this protein *in vivo*, it was fused to the SV5 tag, and used as substrate in the co-transfection assay. As shown in the figure 24B, when is co-expressed with the kinase activator Δ3c and Δ3, SV5-Δ3a substrate is hyper-phosphorylated. On the contrary, the co-expression with Δ3a, does not allow its hyper-phosphorylation. This results confirms that the ability to activate the cellular

kinase maps separated from the region of hyper-phosphorylation that cause the mobility shift. Interestingly, this substrate is clearly less expressed when is not hyper-phosphorylated, as seen in figure 24B, suggesting that hyper-phosphorylation of NSP5 could play a role in the stability of the protein. This was also confirmed when co-transfected with NSP5, while co-transfection of an irrelevant SV5-tagged protein (intrabody anti-ShCH2, an intrabody against N-terminal CH2 domain of the p66 isoform of ShcA) did not have any effect (figure 24C).

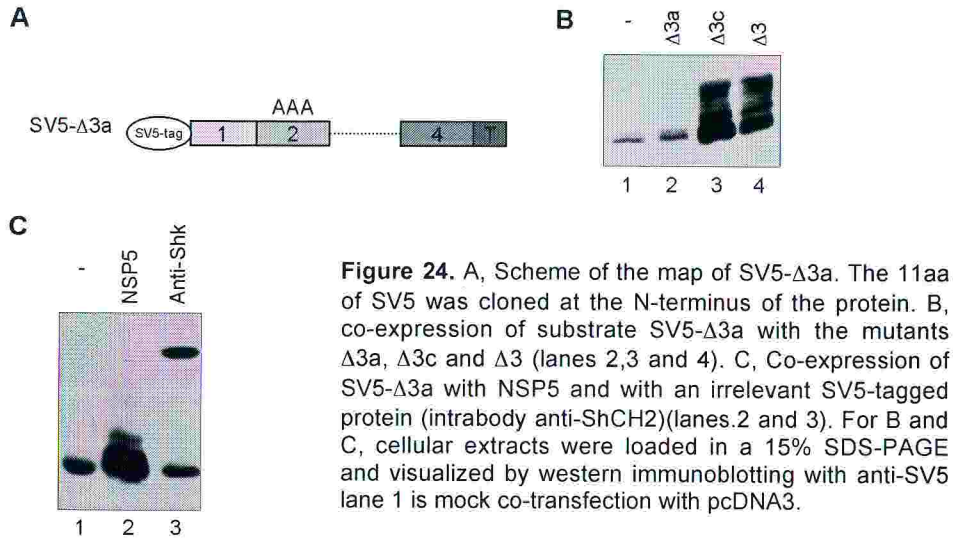


Figure 24. A, Scheme of the map of SV5-Δ3a. The 11aa of SV5 was cloned at the N-terminus of the protein. B, co-expression of substrate SV5-Δ3a with the mutants Δ3a, Δ3c and Δ3 (lanes 2,3 and 4). C, Co-expression of SV5-Δ3a with NSP5 and with an irrelevant SV5-tagged protein (intrabody anti-ShCH2)(lanes.2 and 3). For B and C, cellular extracts were loaded in a 15% SDS-PAGE and visualized by western immunoblotting with anti-SV5 lane 1 is mock co-transfection with pcDNA3.

Localisation to viroplasms: role of serines in motifs *a* and *b*.

Due to the fact that mutant Δ3 fused to EGFP localise to viroplasms (Figure 13), we wanted to study if point mutations in motifs *a* and *b* can affect this property. With this aim, Δ3a, Δ3b and Δ3ab mutants were fused to EGFP at the C-terminus. The assay was performed as described above (see figure 13). The results presented in figure 25 show all mutants localise to viroplasms, indicating that the lack of serine in motifs *a* and *b*, more importantly of serine 67 in motif *a*, did not affect the localisation to viroplasms. A summary of the properties of these mutants including their ability to be substrate and activator are shown in table 5.

Mutations of serines in motifs *a*, *b* or both does not affect the capacity to be substrate in the *in vitro* translation/phosphorylation assay with the activator Δ1/Δ3, indicating that the ability to be substrate is not related to the serines studied in the region 2. On the other hand, we can conclude that the capacity of the mutants to be activator is mainly dependent on phosphorylation of serine 67.

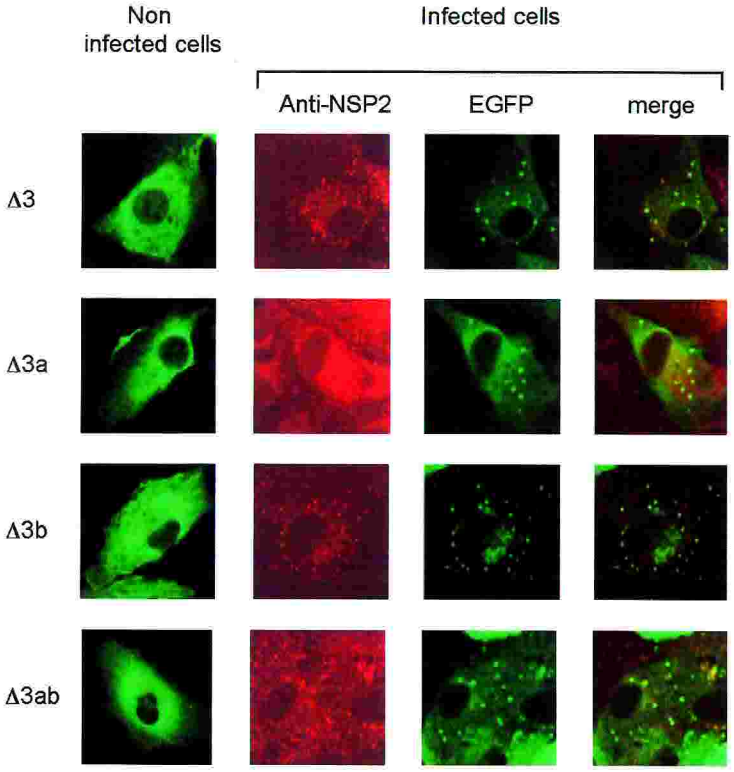


Figure 25. Confocal immunofluorescence. MA104 cells were transfected with Δ3 motifs a and b mutants fused to EGFP followed by infection with rotavirus. Viroplasms were detected with an anti-NSP2 (red) antibody. The rightmost column is the superimposition of the two independently acquired images.

Table 5. Summary of Δ3 mutants properties

protein	Presence of properties[a]				
	Localisation to viroplasms[b]	substrate		activator	
		In vitro[c]	In vivo[d]	In vitro[e]	In vivo[f]
Δ3	+	+	ND	+	+
Δ3a	+	+	+	-	-
Δ3b	+	+	ND	+	+
Δ3ab	+	+	ND	-	ND
Δ3/S67A	ND	ND	ND	-	-
Δ3(S63,65A/S67D)	ND	ND	ND	+	+

[a] +, present;-absent;ND, not determined.
[b] mutant fused to EGFP
[c] *In vitro* translation/phosphorylation assay, Δ1/Δ3 cellular extract used as a source of cellular kinase(s).
[d] *in vivo* hyper-phosphorylation assay: substrate fused to SV5, co-transfected with Δ3 mutants.
[e] *In vitro* translation/phosphorylation assay, NSP5-Δ1 used as substrate.
[f] *In vivo* hyper-phosphorylation assay, SV5-Δ2 used as substrate.

Identification of cellular kinases

We have already shown a series of results suggesting that NSP5 is not a kinase. Some of these evidences are: i) the purified protein His_6-Δ1/Δ3 is not able to hyper-phosphorylate the substrate (figure 8); ii) the GST-NSP5 mutants that are good substrates but are unable to phosphorylate another substrate (figure 9); iii) the fact that basal phosphorylation and hyper-phosphorylation seems to be two separated events. On the other hand, there are some evidences that the phosphorylation and hyper-phosphorylation could be due to a cellular kinase. Such as CK2 or a CK2-like kinase, since: i) there is a characteristic amino acidic region in region 4 homologies to CK2 consensus phosphorylation sites (figure 12) and ii) recombinant CK2 is able to phosphorylated the GST substrate in an *in vitro* experiment CK2. Moreover, as shown above serine 67 present in the region 2 needs to be phosphorylated to activate the cellular kinase for its own phosphorylation. This process of phosphorylation is probable derived from cellular event, characteristic of a cellular kinase. Hitherto, the amino acidic sequence around serine 67 has not been described as a phosphorylation sites for known kinases.

An alternative way to study the cellular kinases involved in the phosphorylation of NSP5 and the role of them during the replicative cycle, is the use of specific peptides as competitors for cellular kinase in the different assays. These kind of peptides are specific for determined kinases, allowing the use of them as specific substrates. Thus to allow identification of the cellular kinases involved in the cascade of phosphorylation. As it was first described, it is possible that a CK2-like kinase plays a role in the hyper-phosphorylation process of NSP5. If this is true, a peptides specific for CK2 should compete and diminish the hyper-phosphorylation of NSP5. For this experiment, it was chosen a specific peptide for CK2 (sequence: RRRADDSDDDDD (252)), which was used at different concentrations in the *in vitro* translation/phosphorylation assay [50-500 µM] with extracts of cells transfected with activator Δ1/Δ3 and the substrates [^{35}S]-Δ1 and [^{35}S]-Δ3 (figure 26A). The plotted results showed a reduction of 50% of the hyper-phosphorylation in the presence of ~150 µM of peptides without a further increase at higher concentration (figure 26B). The same results were obtained for both substrates, indicating that the CK2-like is not the only enzyme involved.

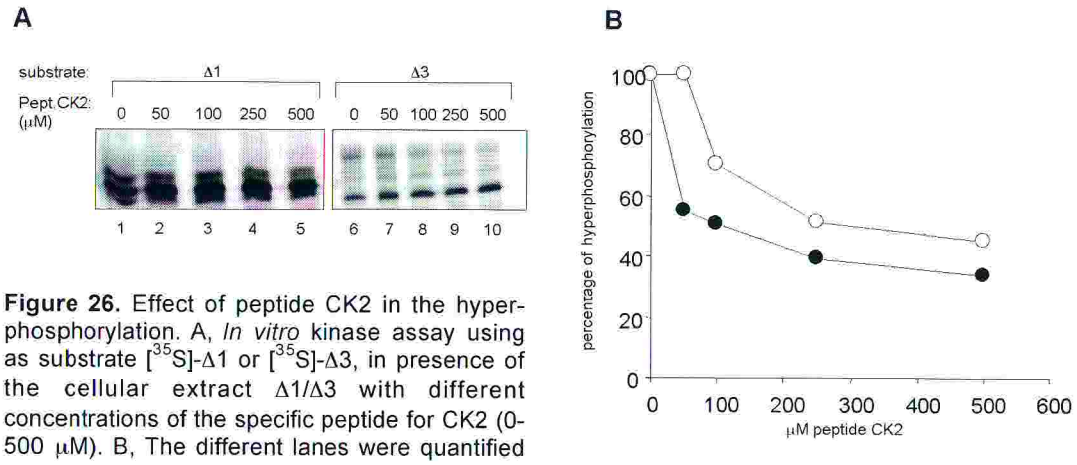


Figure 26. Effect of peptide CK2 in the hyper-phosphorylation. A, *In vitro* kinase assay using as substrate [^{35}S]-Δ1 or [^{35}S]-Δ3, in presence of the cellular extract Δ1/Δ3 with different concentrations of the specific peptide for CK2 (0-500 µM). B, The different lanes were quantified in cyclone densitometer (Packard BioScience Company) and plotted concentration of peptide (µM) versus percentage of hyper-phosphorylation. The substrates were represented as (●) for [^{35}S]-Δ1 and (O) for [^{35}S]-Δ3.

Thus, we analysed the effect of other peptides, specifc for the PKC family. Table 6 indicates the sequences of them. The peptides were selected due to the high specificity. For CK2 it was described that is a ubiquitous serine/threonine protein kinase capable of phosphorylating a wide array of substrates *in vitro*. This enzyme is able to phosphorylated multiple substrates found in various subcellular locations; membranes associated such as the CD5 receptor, cytoplasmic such as IkBα, Drosophila and mouse dishevelled protein or nuclear such as RNA polymerase I and III, TBP, c-Jun, SRF, nucleolin, Antennapedia and ATF-1 (172). Protein kinase C (PKC) family members play crucial roles in the signal transduction of a variety of extracellular stimuli, such as hormones and growth factors. To date, twelve isoenzymes of PKC have been identified in mammalian tissues and subdivided into conventional PKC (cPKC) members comprising α, βI, βII and γ isoforms (activated by calcium, acidic phospholipid and diacylglycerol (DAG)), novel PKCs (nPKC) comprising δ, ε, η, and θ (activated by DAG and acidic phospholipid but insensitive to calcium), and a typical PKCs (aPKC) λ and ζ (mechanism of regulation not clear). Another subgroup of PKCs may be defined by PKCμ, which has a potential signal peptide and transmembrane domain. Since these PKC isoenzymes differ in their expression in different tissues and in their mode of activation, supporting the idea that each isoenzyme may play different roles in signal transduction processes. PKCα is ubiquitously expressed and is activated in response to many different kinds of stimuli and translocates from cytosol to the specialized cellular compartments (nucleus, focal adhesion, caveolae, etc), where it is presumed to work. Therefore, PKCα has been implicated in a variety of cellular functions including proliferation, apoptosis, differentiation, motility and inflammation. However, the responses induced by activation or overexpression of PKCα varies depending on the types and conditions of cells (200). Since PKCμ have very different substrate specificities than others PKCs and is reported to be activated by phorbol esters, this enzyme is likely to mediate novel phorbol ester signalling pathways distinct from those mediated by others PKCs. These two enzymes selected for distinct peptide substrates. PKCα strongly selected for peptides with the basic amino acids Arg

or Lys. In constrast, PKCμ preferred peptides with hydrophobic amino acids (203).

Table 6: sequences synthetic peptides used for assays

Peptide name	specificity	sequence[a]	reference
CK2	K_m 19 μM	RRRADDSDDDDD	(252)
PKCα	K_m 3.8 μM	RRRRRKGSFRRKA	(203)
PKCμ		AALVRQMSVAFFFK	(203)
LAL-4	371-384 aa from human LAL	EWEHLDFIWGLDA	(209)

[a] phosphorylation sites are underlined.

The peptides were tested in a kinase reaction with of the Δ3 mutants in motif *a*, *b* and *c* as [^{35}S]-*in vitro* translated substrates, and of a cellular extracts of Δ1/Δ3 as a source of kinase. The results presented in figure 29, show a clear effect of peptides in each of these mutants. Mutant Δ3 hyper-phosphorylation was very sensitive to peptides specific for CK2, PKCα and PKCμ. However, Δ3 with mutation in the diverse motif were less sensitive with the exception of Δ3c,which was very sensitive at high concentration of peptide for CK2 and PKCα. The use of an irrelevant peptide as LAL-4, proceeding from the human lysosomal acid lipase (209) did not produce any change in the hyper-phosphorylation of all kind of substrates, suggesting that peptides work specifically. However, none of these results produced a conclusive answer to the specificity of the cellular kinase, and thus, its identification still remains obscure.

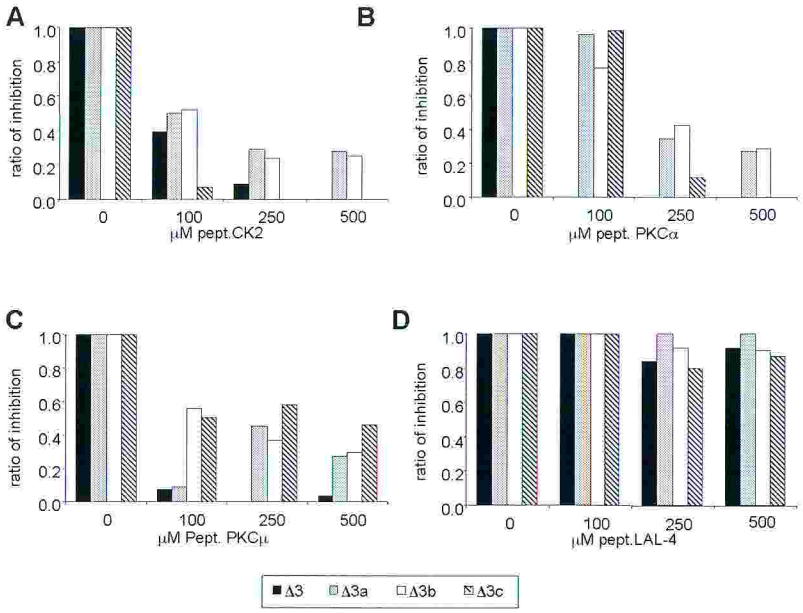


Figure 27. Comparison of different specific peptides in the hyper-phosphorylation of Δ3 substrates. Kinase reaction were performed in presence of specific peptides for the kinases CK2 (A), PKCα (B) and PKCμ (C), as control peptide was used LAL-4 (D) [0-500 μM]. *In vitro*-translated substrates labelled with [^{35}S] for Δ3, Δ3a, Δ3b and Δ3c were used. The cellular extract transfected with Δ1/Δ3 was used as enzyme. The samples were loaded in SDS-PAGE 15% and exposed in cyclone densitometer. The radioactive signal were measured and plotted as concentration of peptides [μM] versus ratio of inhibition, in which ratio is A/B, where A represented the peptide concentration [0-500μM] and B represented the peptide concentration at [0 μM]. A and B were calculated in function at the next equation; [(hyper-phosphorylation/total phosphorylation x 100)-background], where: total phosphorylation = hyper-phosphorylation + basal phosphorylation; (background = hyper-phosphorylation/total phosphorylation x 100) when: peptide is [0 μM] and mock cellular extract.

NSP5/NSP2 interaction and localisation to viroplasms

Localization to viroplasms.

We have previously demonstrated that NSP5 and NSP2, in addition to the interaction observed in virus infected cells, are able to associate *in vivo* in the absence of other viral proteins to form viroplasm-like structures (VLS). With the aim of investigating the localisation of both proteins to viroplasms, we constructed NSP2-EGFP and NSP5-EGFP fusion proteins (in both cases at the N-terminus of EGFP) and used them to obtain stable transfectants that were subsequently infected with rotavirus. As shown in figure 28A, virus infection induces a rapid re-distribution of the fusion protein, with localisation in viroplasms. However, while NSP2 appears to occupy the central part of the viroplasms (figure 28B), NSP5 localises in a more external part as judged by the ring structure formed. This result was obtained with either NSP2 or NSP5 fused to EGFP and the corresponding partner protein visualised with a specific antibody. In addition, similar results were obtained in virus infected cells using antibodies specific for NSP2 (green) and NSP5 (red) that showed part of the two proteins co-localising while a ring of NSP5 was still visible (not shown).

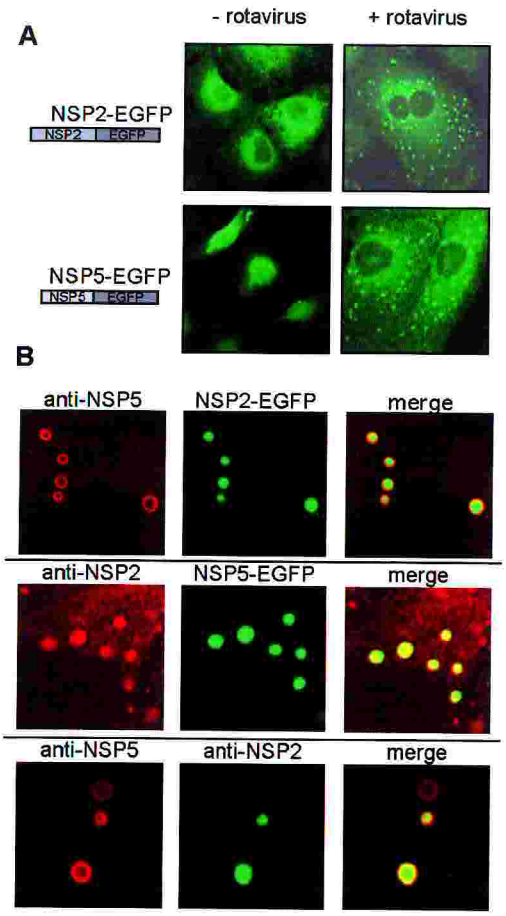


Figure 28. A, MA104 cells stable transfected with NSP2-EGFP or NSP5-EGFP, were infected with rotavirus and analysed at 4h post-infection. B, Amplified confocal images of viroplasms in rotavirus infected cells. Upper panel, cells expressing NSP2-EGFP and reacted with anti-NSP5 sera (red); middle panel, cells expressing NSP5-EGFP and reacted with anti-NSP2 serum (red); lower panel, double immunofluorescence in infected cells reacted with anti-NSP5 (red) and anti-NSP2 (green). Images were obtained by confocal microscopy.

Kinetics of viroplasms formation

We took advantage of the NSP2-EGFP stable-transfected cells to follow, by confocal microspcopy, the assembly of viroplasms at different times post-infection (from 2 to 24 hours). Infection was also assessed by immunofluorescence with a specific anti-NSP5 antibody (red) (figure 29A). The number and area of viroplasms was counted using the overlay programme. As shown in figure 29B, while the area of single viroplasms increased with time, especially after 6 h of infection, the total number of viroplasms per cell diminished. These results suggest a fusion between different viroplasms that could be represented by some of them appearing with a bean shape (figure 29). In addition, a three-dimensional reconstruction of stacked images obtained at different levels in the horizontal plane (figure 30) suggested that viroplasms are spherical structure.

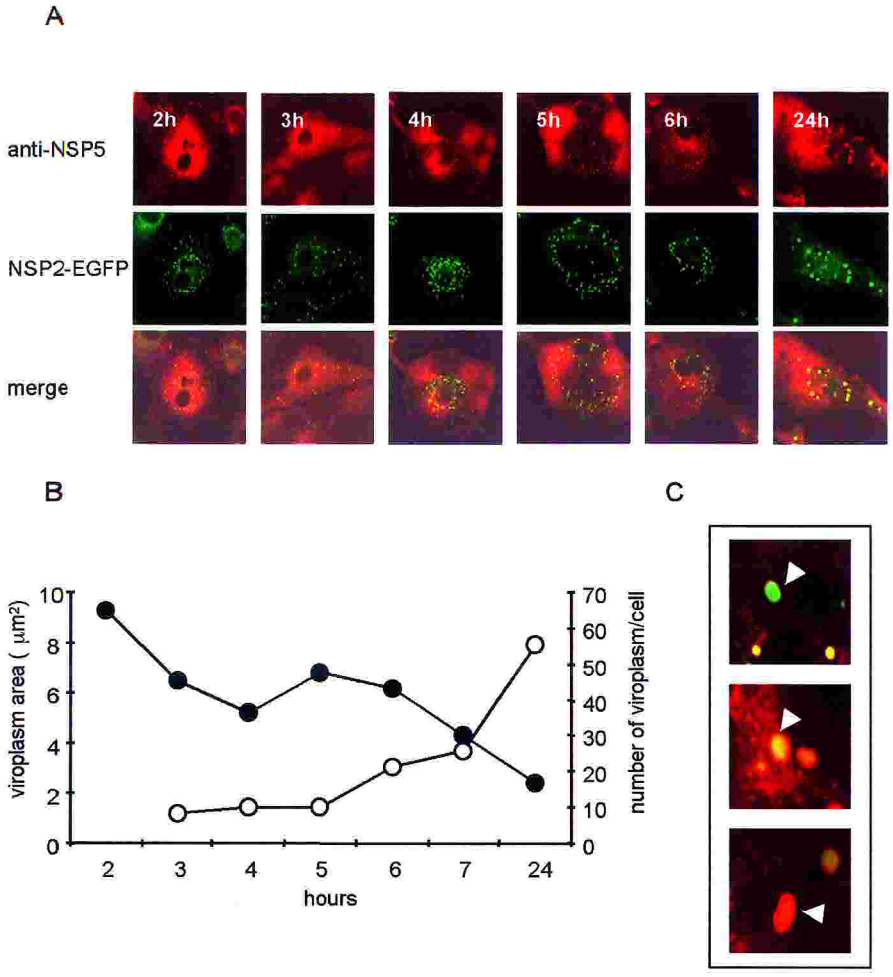


Figure 29. A, Viroplasm visualised by confocal microscopy at different times post-infection, in cells expressing NSP2-EGFP and infected with rotavirus. NSP5 was detected with anti-NSP5 serum (red). B, Plot of the number and average area of viroplasms, determined at different times post-infection. Each time point corresponds to the average of 20 cells counted. (O), viroplam area, (●) number of viroplasm. C, Viroplasms at 6 h post-infection with bean shape (arrow).

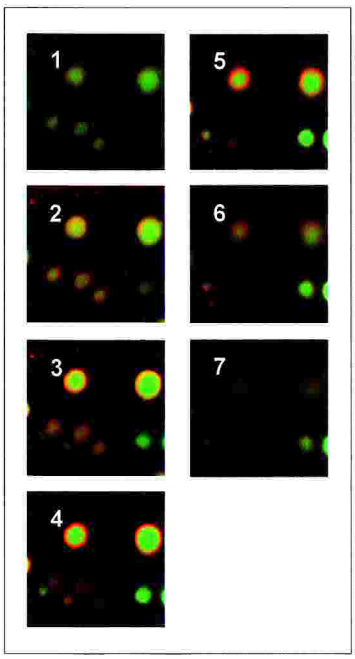
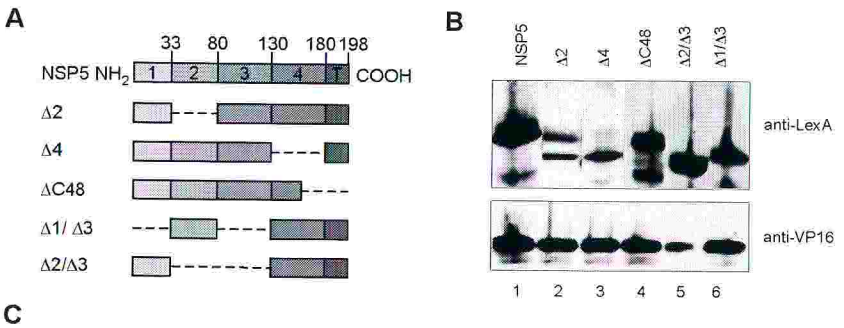


Figure 30. Horizontal stack images of viroplasms in cells expressing NSP2-EGFP and rotavirus infected infected, at 24 h of post-infection. NSP5 is visualized in red. Numbers indicate the order of the stacks.

Two-hybrid interaction between NSP5 mutants and NSP2.

In order to further characterise the NSP2/NSP5 interaction we used the two-hybrid assay to identify the relevant NSP5 domains involved. The NSP5 deletion mutants represented in figure 31A, described in refs. (4, 77) and figure 7 were used as baits, with NSP2 fused to the herpes simplex virus trans-activator protein VP16. (283). Positive interaction was determined by growth in a medium lacking histidine followed by β-galactosidase assay. All baits used in this assay were first tested to rule out a possible trans-activation activity in the absence of NSP2 and their expression checked by western blot (figure 31B). For instance wtNSP5 as well as mutant Δ3 could not be used in this assay because they showed transactivating activity *per se* (see below).

The results obtained are shown in figure 31C. Mutants with regions 2 or 4 deleted can interact with NSP2, while mutants in which region 1, 3 or the C-terminal T were deleted cannot. These results indicate that the N- and C-terminal regions as well as the central part (region 3, aa 81-130) of NSP5 play a relevant role in the interaction with the NSP2.



C

bait LexA	Interaction with NSP2-VP16
Δ2	+++
Δ4	+++
ΔC48	-
Δ1/Δ3	-
Δ2/Δ3	-

Figure 31. A, Scheme of the NSP5 mutants used in the two-hybrid assay. B, Western immunoblotting of (baits NSP5 mutants fused to LexA), with an anti-LexA serum (upper panel) and fish (NSP2 fused to VP16), with an anti-VP16 serum(Lower panel. C, Results of the interaction between NSP5 mutants and NSP2. +++, Cells grew in plates lacking histidine and were positive to the β-galactosidase assay. -, Cells did not grow in plates lacking histidine and were negative to the β-galactosidase when grown in histidine.

Binding assay in mammalian cells.

As an alternative method for studying NSP5/NSP2 interaction we performed an immunoprecipitation assay from total cell extracts expressing NSP2 and various NSP5 mutants. For this purpose we constructed an inducible recombinant vaccinia virus for NSP2 (rVV/NSP2) (286) As shown in figure 32A, expression of NSP2 was obtained from IPTG induced MA104 cells infected with the rVV/NSP2. A single band (approximately 35 kDa) with SDS-PAGE mobility identical to NSP2 from rotavirus strains OSU and SA11 was obtained.

To analyse the binding of NSP2 to NSP5, cells were infected with rVV/NSP2 at a multiplicity of 3 PFU/cell, transfected with the different NSP5 deletion mutants, induced with 1 mM IPTG and labelled overnight with [^{35}S]-methionine. To stabilize the interaction, chemical crosslinking with DSP was performed in living cells, followed by lysis and immunoprecipitation with anti-NSP5. The relative binding was determined by densitometry of the bands obtained following autoradiography of the SDS-PAGE. The ratio was calculated as described in materials and methods.

A representative binding assay is shown in figure 32B for some of the mutants. As expected, no cross-reactivity was observed in immunoprecipitations with anti-NSP5 in cells that were only infected with rVV/NSP2 and expressed no NSP5 (lane 1). In figure 32C, it is shown the relative binding of the different mutants, with respect to wtNSP5, which was taken as a reference value of 1. Each NSP5 deletion mutant experiment was performed three times and a mean value was calculated. Deletion of region 1, like mutants Δ1, Δ1/Δ2 and Δ1/Δ3 has a profound effect in the ability to bind NSP2. Similarly, deletion of the C-terminal region T (mutants ΔT, Δ4T, Δ2/Δ4T and Δ3/ΔT) also produced strong binding impairment. These results suggested, in agreement with the two hybrid experiments, that the N- terminal and the C-terminal tail, are relevant for NSP2 binding. Moreover, independent deletion of other regions (like regions 2, 3 and 4) suggested that they are not directly involved. Interestingly, region 3 appears to have an inhibitory effect. In fact, deletion mutant Δ3 showed a relative binding two fold higher than wtNSP5. However, when we deleted regions 1 or T from this mutant (like Δ1/Δ3

or Δ3/ΔT), NSP2 binding was completely abolished. Thus, it seems that region 3 is not related with binding activity.

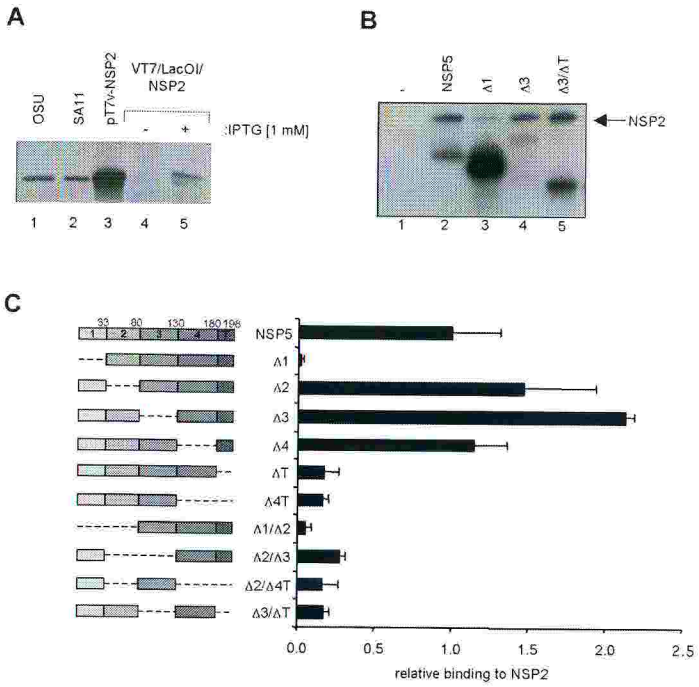


Figure 32. A, Immunoprecipitation with anti-NSP2 sera of cellular extracts, from [^{35}S]-methionine labelled MA104 cells, infected with rotavirus OSU (lane 1) or SA11 (lane 2) or with vaccinia virus VVT7 and transfected with pT7v-NSP2 (lane 3), or recombinant rVV/NSP2, non induced (-, lane 4) or induced (+, lane 5) with 1 mM IPTG. B, *In vivo* binding assay. Electrophoresis of extracts of cells infected with 3 MOI of rVV/NSP2, transfected with the indicated NSP5 mutants, labelled with [^{35}S]-methionine and immunoprecipitated with anti-NSP5. C, NSP2 binding activity of NSP5 mutants. plotted relative to wtNSP5, taken as 1. The average value for each mutant was obtained from three independent assays.

Viroplasm localisation is dependent of regions 1 and T.

To further demonstrate that the N- and C- terminal regions are indeed the only ones required for interaction with NSP2 and localisation to viroplasms, we turned to new constructs, in which region 1 was fused at the N-terminus of EGFP and regions 4, T or both, at the C-terminus. The different constructs, schematically shown in figure 33, were used to study formation of VLS by co-transfection with NSP2 and viroplasm localisation in virus infected cells. VLS were only obtained with variants containing both region 1 and T, while localisation to viroplasms could be seen even when region 1 was not present like in EGFP-4T. This is likely the consequence of EGFP-4T interaction with viral NSP5 that depends on NSP5 C-terminal residues (276) and Figure 13.

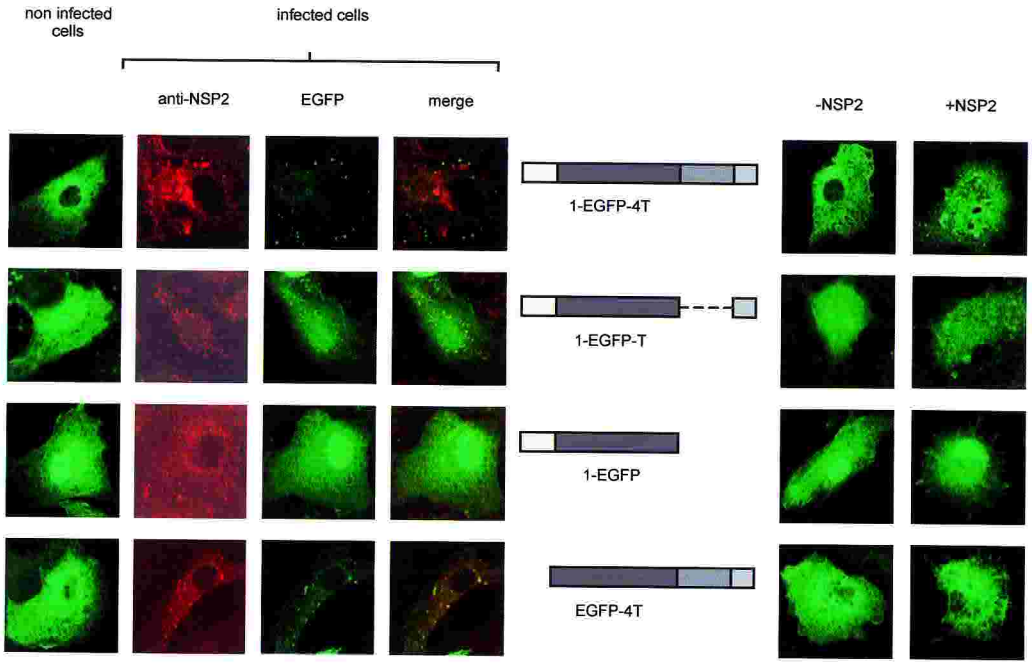


Figure 33. Viroplasm localisation and VLS formation of EGFP fused to the N-and C-terminal regions of NSP5. Viroplasm localisation was determined at 4 h post-infection (left panel), while formation of VLS was determined at 18 h post-transfection of cells infected with rVV/NSP2 and immediately transfected with the indicated constructs.

In summary, Table 7 shows the interactions mapped on NSP5 in terms of localisation to viroplasm of NSP5 mutants fused to EGFP and of VLS formation with NSP2. These results are consistent with the ones obtained with the two-hybrid analysis and the *in vivo* binding assay, in which the N-terminal region and the C-terminal tail are the two regions necessary for NSP2 binding.

Table 7. Interaction of NSP5-EGFP mutants.

Mutants	Localisation to viroplasm[b]	VLS[c]
1-EGFP-4T	+	+
1-EGFP-T	-/+	-
1-EGFP	-	-
EGFP-4T	+	-

[a] +,present;-, absent;±, marginal.
[b] cells infected with rotavirus.
[c] co-transfection with NSP2.

DISCUSSION

Rotavirus NSP5 is a protein without a clear role in the virus replicative cycle. This protein presents two different post-translation modifications that could be related to its function. One of this post-translational modification is an O-linked N-acetylglucosamine (O-GlcNAc) glycosylation, a particular kind of glycosylation that is present in proteins localized in the cytoplasmic and nucleoplasmic compartments of the cell. This glycosylation is present in NSP5, isoforms of 26 and 28 kDa (102) and much less relevant (if any at all in other higher MW isoforms). These isoforms correspond to phosphorylation that heavily affects NSP5. Indeed, NSP5 was originally described as a phosphoprotein (291) and later demonstrated to replace in serine and threonine (5, 25). In part, NSP5 phosphorylation modifies NSP5 generating different phosphorylated isoforms with apparent MW ranging from 26 to 32-34 kDa. Interestingly, the above mentioned O-glycosilation occurs also is serine and threonine has been proposed to play a role in regulating phosphorylation sites (113). As mentioned in the introduction, during the infective cycle NSP5 interacts with NSP2, VP6 and VP1. When NSP5 is expressed only with NSP2 in absence of others viral proteins, it produces a pattern of phosphorylation that resembles the one obtained in virus infected cells (4, 5, 224). This was true for, NSP5 expressed from both the SA11 sequence and the OSU sequence. This is important because the lower encodes also for NSP6, while the latter has only a very short ORF of 51 aa. With the aim of studying the phosphorylation of NSP5, in our laboratory were constructed a series of deletion mutants. Some of them present a pattern of hyper-phosphorylation similar for the one found in the infective cycle. Among the deletion mutants with this characteristic are those in which regions 2, 4 and T are present (77). To further understand the details of the mechanism of the phosphorylation of NSP5 and its role in the function of the protein, other mutants were constructed and analysed in alternative assays, both *in vitro* and *in vivo*.

NSP5 is not a kinase?

As described in the introduction a protein phosphorylation involves the enzyme–catalysed transfer of the terminal phosphate group of an ATP(or GTP) molecule to the hydroxyl group on a serine, threonine or tyrosine side chain of the protein. This reaction is catalysed by a protein kinase, and the reaction is essentially unidirectional because of the large amount of free energy released when the phosphate-phosphate bond in ATP is broken to produce ADP. Cells contain hundreds of different protein kinases, each responsible for phosphorylating a different protein or set of proteins. The protein kinases that phosphorylate proteins in eucariotyc cells belong to a very large family of enzymes, which present an active site, where ATP and peptide to be phosphorylated are held, a phosphate-binding loop and a catalytic loop corresponding to a sequence of 250 amino acids (141).

NSP5 until now has been described as a phosphoprotein and, in some reports, with autokinase activity. The evidences that support that this viral protein is a kinase are very poor and are not consistent with the characteristics described for eukaryotic kinases. The autophosphorylation of NSP5 from cellular extracts are not sufficient evidences to classify this protein as a kinase (4, 26, 27, 276). Recent computational analysis using the kinase sequence resource KSD website (http://kinase.ucsf.edu/ksd/data.html) (38), in which it was made an assignment of sequence to homologous kinase family with rotavirus NSP5 strain OSU. The results obtained generated a 'low homology score without much significance'. This suggested that NSP5 has not a characteristic kinase catalytic domain. The closest homology was found with family kinase GSK3-II (family 175). The philogenetic tree shows clearly that NSP5 (GI#139554) is very distant to the other member kinases of family 175 classified with low homology score (figure 34).

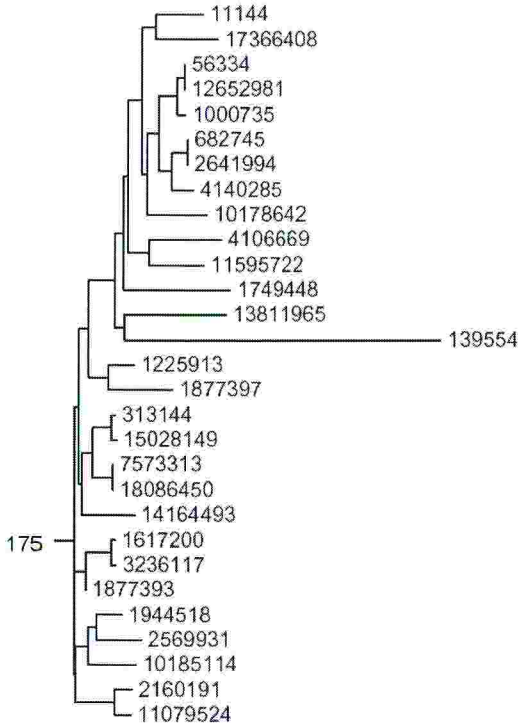


Figure 34. Genetic relatedness between NSP5 (139554) and members of kinase family number 175. Date obtained from KSD website.

In addition, in several attempts purified GST-NSP5 and $(His)_6$-NSP5 were always unable to show kinase activity, both to themselves or *in trans* to another NSP5 molecule. During the purification process of $(His)_6$-Δ1/Δ3, protein was washed in a mild (150 mM NaCl) form in which, the activity of phosphorylation was maintained, but stringent washes (400 mM NaCl) the phosphorylation activity was loss, indicating that probably Δ1/Δ3 remain in interaction with a cellular kinase and by an increment of the ionic charge this interaction is disrupted. The fact, that some NSP5 mutants with multiple deletions, as Δ1, Δ3 or Δ1/Δ3, have a hyper-phosphorylated pattern and the molecular masses of these proteins is around 130 KDa is another element to suggest that NSP5 is not kinase but, an activator. The separated functions attributed, related to phosphorylation, to NSP5 as activator and substrate (in regions 2 and 4, respectively) demonstrate that a catalytic domain or a nucleotide-binding site are not present in this molecule. Moreover, NSP5 has not the characteristics required to have a catalytic domain. As described by Taylor *et al.*, 1992 (272), the catalytic loop from kinases in general, has conserved amino acids, as region IV (loop between two β-strands)

with invariant residues, Asp and Asn, in which Asp help to facilitate the rapid release of the phosphopeptide once phosphotransfer has taken place. Asn lies on the other side of the loop, and its side chain serves to stabilize the loop by hydrogen bonding to the backbone carbonyl of the Asp (above). Also, Arg and Lys are highly conserved, in which especially Lys is conserved in Ser/Thr kinases. An alignement of the predicted secondary structures, using jpred server [http://www.compbio.dundee.ac.uk/~www-jpred/], of NSP5 from different rotavirus strains, indicate that is not a conservation between the β-strands and the amino acidic residues, above mentioned, are not conserved. This is a clear indication that NSP5 has not a catalytic domain.

Characterisation of the autoregulation of NSP5 hyper-phosphorylation.

In this thesis, it was developed an *in vitro* translation/phosphorylation assay consisting in the use of a *in vitro* translated NSP5 mutant labelled with ^{35}S-methionine, as substrate, and total extracts from cells transfected with NSP5 mutant as a source of kinase. This assay was the consequence of a previous observation reported by Afrikanova *et al*. 1996., in which *in vitro* translated NSP5 was able to be phosphorylated producing mobility shifted forms, only when incubated with extracts from virus-infected cells, but not from non-infected cells. We used this assay with NSP5 deletion mutants to discriminate regions of NSP5 necessary for the substrate activity as well as the requirements for the cellular extract to have the kinase activity able to produce PAGE mobility shift.

We found that cell extracts containing NSP5 deletion mutants lacking either domain 1, 3 or both (Δ1, Δ3 or Δ1/Δ3, respectively) efficiently instruct PAGE mobility shift to an *in vitro* translated NSP5-derived substrate. Consistent with these results, these same mutants were previously shown to undergo mobility shift phosphorylation *in vivo* (77). Similarly, cell extracts of mutants lacking regions 2, 4 or T, that were inactive in the assay were unable to be phosphorylated *in vivo* (77) in spite of the fact that two of them (Δ2 and ΔT) contain region 4. Interestingly, a cellular extract of cells transfected with the

histidine-tagged version of mutant Δ1/Δ3 (His_6Δ1/Δ3) showed high activity, while the purified protein did not. Moreover, GST fusion proteins showed complete lack of kinase activity, whereas they were good substrates. Taken together these results indicated that NSP5 is not itself a kinase, but rather it activates a cellular kinase(s) for its own phosphorylation, otherwise inactive in the untransfected cellular extracts.

The marginal phosphorylation activity of extracts containing wild type NSP5 is not surprising since, when expressed alone, NSP5 is very little phosphorylated producing mainly the 26 kDa polypeptide (4, 5) while, upon co-expression with NSP2 it becomes hyper-phosphorylated (4). Regions 1 and 3 appear to play an inhibitory role (region 3 being stronger than region 1, figure 8A) in the capacity of NSP5 to activate the cell kinase(s), as revealed by the activity of mutants Δ1, Δ3 and Δ1/Δ3. One could speculate that in virus–infected cells, interaction with NSP2, may neutralise the inhibition of these two regions.

Serines 153, 155, 163 and 165 can be CK2-like substrates

Region 4 contains the main phosphoacceptor sites, which we mapped to serines 153, 155, 163 and 165, located in an acidic region, with homology to CK2 phosphorylation sites (SDSE and SDSD) (171). In fact, we showed that CK2 was able to phosphorylate NSP5 *in vitro* precisely in those positions, although this does not demonstrate that CK2 is the kinase responsible for NSP5 phosphorylation *in vivo*, nor that other kinases may not be involved. These results suggest that the cellular kinase(s) activated by NSP5 is a CK2-like enzyme. Up to now CK2 has been described to phosphorylate several viral proteins of both DNA and RNA viruses as: a) phosphoprotein P from RSV, b) protease (PR) from HIV-1, c) NS5A from HCV, d) NSs from rift valley virus, e) movement protein (MP) from tomato mosaic tobamovirus, f) gE from HSV-1 and g) tegument protein from HSV-1 (68, 112, 140, 142, 178, 191, 194), with the exception of viruses of the *Reoviridae* family.

Noteworthy, phosphorylation of the four serines in region 4 appears not to be required for kinase activation since mutant Δ1/Δ3 (Ser→Ala) was still able to induce the cellular kinase(s).

The *in vivo* co-transfection/ phosphorylation assay

Serine 67 is essential for kinase activation

According to the *in vitro* studies we hypothesised that NSP5 hyper-phosphorylation was a consequence of the activation cellular kinase by NSP5 itself, involving regions 2, 4 and T which appeared to be essential. In order to obtain more relevant information of the process we developed an *in vivo* alternative assay. We reasoned that, if NSP5 activates a cellular kinase, then it should be possible to separate the activation from the substrate activity. We thus decided to perform co-transfections. Since region 2 was apparently determinant for kinase activation, we decided to investigate if phosphorylation of serine in this region could be involved in such process. Of these serines, six were selected due to the amino acidic context in which they are present. That, we named as two motifs, *a* and *b* corresponding to serines 63, 65 and 67 and to serines 73,75 and 78, respectively. To study these serines, they were point mutated to alanine in mutant Δ3. We showed point mutations in serines motif *a* obstructed two different functions its own hyper-phosphorylation and its cellular kinase activation. A thorough analysis of the three serines present in this motif *a*, revealed clearly that serine 67 was responsible for the lack of activity. This was confirmed with the mutation from serine to aspartic acid (this amino acid is able to mimic a serine phosphorylated, due to the length and the negative charge of the R group (42, 123). The results strongly suggested that a phosphate in serine 67 was required to restore kinase activation and ability to be hyper-phosphorylated. More importantly, when these mutations (S63A, 65A and S67D) were introduced in the full-length NSP5, the protein gained the ability to become hyper-phosphorylated, a property that was not present in the wtNSP5 alone but that is characteristic of NSP5 in virus-infected cells. On the other hand, it was described that the presence of NSP2 produces a hyper-phosphorylation of

NSP5. This situation can be verified when these two proteins are co-transfected in the absence of other viral proteins (4). As expected, co-expression with NSP5a did not produce its hyper-phosphorylation, while NSP5(S63,65A/S67D) was hyper-phosphorylated even in the absence of NSP2. These results together with the fact that deletion mutants Δ1, Δ3 and Δ1/Δ3 are hyper-phosphorylated *in vivo* in absence of other viral proteins (4, 77), suggested that NSP5 hyper-phosphorylation and kinase activation function is due to conformational changes in NSP5 produced by the interaction with NSP2 a situation that can be mimicked by deletion of regions 1, 3 or both, which play an inhibitory effect. In this scenario, serine 67 becomes available for phosphorylation by a cellular kinase(s), and this event makes the protein an activator of a kinase that phosphorylates region 4.

However, not only serine 67 phosphorylated has a crucial role in the hyper-phosphorylation process of NSP5. As shown in the result, the lack of the tail (T) in deletion mutants with well established kinase activation function, like Δ1 and Δ1/Δ3 obstructed their capacity to hyper-phosphorylate the substrate SV5-Δ2. As described by Torres-Vega *et al.*, 2000 (276), the last C-terminal 10 amino acids of NSP5 appear to be involved in dimerisation with another NSP5 molecule. The sequence that we called T is constituted by 18 aa, and by silicon analyses it appears to have a secondary structure [(PHDsec), http://www.embl-heidelberg.de/predictprotein/predictprotein.html)] of a α-helix. This secondary structure has a high probability to dimerise through the formation of complementary charge interactions between two α helices (32). With these evidences, we can reasonably suggest that the relevance of tail resides in the dimerisation *in trans* of the activator molecule with another NSP5 (substrate) molecule, thus allowing hyper-phosphorylation.

The role of region 4 appears to be important mainly as the site of phosphorylation rather than in the activation, as shown by the capacity of mutant Δ2c to be an activator but a poor substrate. We suspect that in region 4, others serines such as serine 137 and 142 could be involved in the hyper-phosphorylation.

Taken together all the evidences, we propose a model for the NSP5 hyper-phosphorylation. In this model, as shown in the figure 35, an NSP5 molecule interacting with NSP2 is phosphorylated in serine 67 by a cellular kinase. This phosphorylation is essential for the protein to become an activator of another cellular kinase probably by physical interaction of the region 2, phosphorylated in serine 67 with an activation domain of the kinase. At this moment the dimerisation *via* the region T *in trans* with another NSP5 molecule occurs, which can then interact with the phosphorylation domain and become hyper-phosphorylated. This interaction is most likely involving the phosphorylation domain of the kinase and region 4 of NSP5.

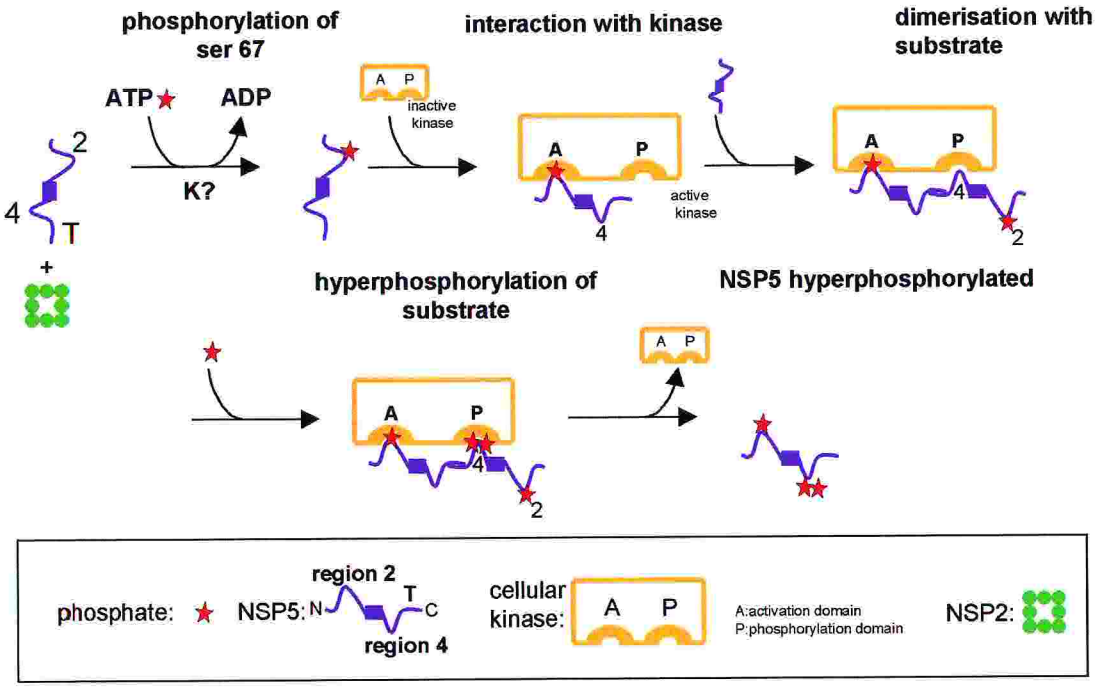


Figure 35. Model for NSP5 hyper-phosphorylation. After the interaction with NSP2, the NSP5 hyper-phosphorylation is due principally by activation of a cellular kinase by a interaction with region 2, where is present the phosphorylated serine 67. Later, a dimerisation with by tails *in trans* with new NSP5 molecule, in which this can interact by the region 4 allowing the hyper-phosphorylation process. The scheme of phosphate, NSP5 molecule and the cellular kinase are indicated.

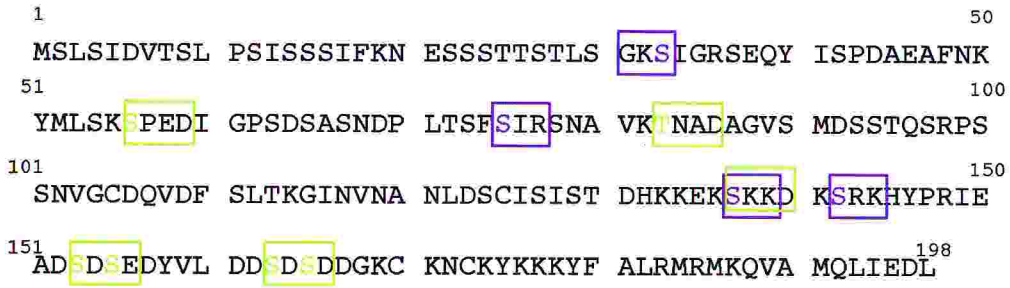
In this model, as verified experimentally, a key player of the regulation of NSP5 hyper-phosphorylation, is NSP2 (4). NSP2, that is a RNA-binding, NTPase, RNA helicase and able to form octamers (125, 138, 253, 267-269), could be considered as a molecular matchmaker. These are defined as proteins that, in an

ATP-dependent reaction, bring two compatible yet solitary macromolecules together to promote their association, and then leave the complex (248). This mechanism of action is characteristic of a broad range of matchmaker. In this case, "matchmakers" are not phosphorylated themselves by protein kinases. They directly interact with their substrates, changing their conformations and biochemical properties. An appropriated mechanism to describe the role of this kind of molecule is: 1) in the absence of the matchmakers, the target molecule is constrained in the "closed form" and only certain phosphorylation sites are available to kinases. 2) When, matchmakers bind the target molecule changing its conformation to the "open form", if the target molecule is a substrate for protein kinases, new phosphorylation sites are made available or, the existing sites are made more accessible. In some cases, this leads to the hyper-phosphorylation of the target molecule. Examples of this kind of interaction have been described in HCV (Hepatitis C virus) for proteins NS3 and NS5A, where the interaction with NS3 enhances the hyper-phosphorylation of NS5A. This event is common for all HCV strains and for other members of the family *Flaviridae*, implying its importance in the life cycle of these viruses (254). In rotavirus however, although the interaction with NSP2 allows NSP5 hyper-phosphorylation, it cannot ruled out that during the infective cycle, others viral proteins like VP1, VP2 and VP6, or currently unknown cellular proteins could also participate.

Kinases that can be implicated in the NSP5 phosphorylation.

The proposed model can explain how is the process by which NSP5 becomes hyper-phosphorylated. The complex phosphorylation pattern of NSP5 during the infection, suggested that regulation is due to distinct phosphorylation events. However, the cellular kinases that are involved in this process remain unknown. As firstly described, the four serines of motif *c* in region 4 have an amino acidic context, which can be phosphorylation sites for CK2. Indeed, we showed that recombinant CK2 can also phosphorylated NSP5. The use of specific peptides as competitors for the kinase with a determined substrate, is a valid approach to identified which cellular kinase are involved in the phosphorylation processes. In

this thesis were used peptides specific for CK2 (252), PKCα and PKCμ (203). The choice of these peptides was due for silicon analysis (PPSEARCH/Prosite, http://www.ebi.ac.uk) in which NSP5 showed putative phosphorylation sites for CK2 and PKC (figure 36). These studies with peptides indicated that they had an effect in the hyper-phosphorylation, but the competition was not complete, thus not providing conclusive results. CK2 has a relevant role in the phosphorylation of several viruses. PKC has also been described to be involved in the phosphorylation of some viruses, like the phosphorylation of protein P from rabies virus, the regulation of DNA-unwinding activities of the NS1 from minute virus mice (57, 110, 204). In NSP5, serines 153, 155, 163 and 165 are putative phosphorylation sites for CK2, while serines 137 and 142 are putative sites for PKC. On the other hand, as shown in figure 36, until now the motif where serine 67 is inserted, was not being recognized as a phosphorylation site by any kind of kinase. Personal communication with Dr. J. Allende proposed that, serine 67 could be a non-classical phosphorylation site for CK1. This alternative is under investigation.



[a]violet rectangle are predicted phosphoryaltion sites for PKC
[b]green rectangle are predicted phosphorylation sites for CK2
[c]Ser and Thr that phospho-sites are indicated
[d]Prediction obtained by PPSEARCH/Prosite (http://www.ebi.ac.uk)
[e]Protein databank number:628279

Figure 36. The sequence of porcine NSP5 OSU was analysed using the Prosite system. In this form were predicted phosphorylation sites for PKC and CK2.

An important aspect which appears to be related with the NSP5 phosphorylation is the stability of the protein. For instance in the case of Δ3a, which is not phosphorylated is not stable when is expressed alone. However, the co-

expression with a mutant with kinase activator function allows this mutant to become hyper-phosphorylated, but also to increase the amount of protein, suggesting that the phosphorylation could be directly related with the stability of NSP5. Phosphorylation and proteins stability has also been shown for other viral proteins. It was described that, the MP protein (movement protein) from TMV (tobacco mosaic virus) requires to be phosphorylated in serine 37. Mutation of Ser 37 to Ala, Glu or Thr dramatically affected the association of MP with ER, microtubules, and plasmodesmata, as well as stability of the protein (18). On the contrary, the NS5A from HCV, that as a hyper-phosphorylated isoform seems to be more labile in general (285). It remains to be determined the pathway of NSP5 degradation and the way in which phosphorylation affects its stability.

Structure of viroplasms and interaction with NSP2.

The non-fusiogenic mammalian orthoreovirus replicate and assemble in cytoplasmic phase-dense inclusions in infected cells. These inclusions contain viral double-stranded RNA (259), viral proteins, and partially assembled viral particles (63, 238). In the case of rotavirus, the inclusion bodies, called viroplasms, are globular structures identified as the machinery of virus replication. Immunogold staining with antibodies against NSP2 and NSP5 suggested that both proteins localised in the external part of the globular structure of the viroplasms (217). The interaction of NSP2 with NSP5 was first described *in vivo*, in rotavirus infected cells, by UV or chemical (DSP) crosslinking. Both treatments stabilised the interaction between the two proteins (4). However, other experiments demonstrated the interaction between the two proteins, independently of the viral context, when co-expressed in MA104 cells in the absence of all the other viral proteins, forming spherical viroplasms like structure (VLS) that were not observed when each protein was expressed individually (77, 224). Many questions concerning phosphorylation of NSP5 and its ability to localise in viroplasms await the development of a reverse genetic system.

The formation of VLS reflects the ability of the two non-structural proteins to interact with each other. On the other hand, in the context of viral infection when using NSP5 deletion mutants, the situation is more complex since localisation to viroplasms can also implicate interaction of the transfected mutants with wild type NSP5 (276). Contrary to what was observed with VLS formation (77), all NSP5 mutants containing regions 4 and T localised to viroplasms independently of the phosphorylation state. Interaction with wild type NSP5 could explain this behaviour, since dimerisation/multimerisation of NSP5 was shown to depend on the last ten carboxy-terminal amino acids (276). In fact, mutant Δ2 does not get phosphorylated when expressed alone *in vivo* and does not form VLS (77), whereas in this thesis we found localisation to viroplasms. These results could be interpreted on the light of the hyperphosphorylation of these mutants in the presence of the activated cellular kinase (which is obviously present in virus infected cells). Consistent with this, we finding that wtNSP5 as able to complement Δ2 VLS formation, in experiments of co-transfection of Δ2-EGFP with NSP2 in the cell line stably expressing NSP5 (C7). All these results suggest that indeed phosphorylation may be required for proper localisation to viroplasms. In this line mutant Δ4 showed a viroplasms negative phenotype. However, either the basal or hyper-phosphorylation of NSP5 could also be required for interaction with other proteins. In the fact, NSP5 mutated in motif *a* still has a basal phosphorylation level.

In this thesis, we used confocal microscopy to investigate the distribution in viroplasms of NSP2 and NSP5. For this purpose, we produced stable transfectants of NSP5 or NSP2 fused to the N-terminus of EGFP that presented a difused cytoplasmatic distribution. Following rotavirus infection each non-structural fusion protein showed complete re-localisation to viroplasms. Interestingly, upon magnification, it appears clear that NSP5 resides in a more external region with respect to NSP2. This result is in agreement with co-immunoprecipitation experiments carried out with anti-NSP2 or anti-NSP5, which showed that, while anti-NSP5 was able to co-precipitate NSP2, anti-NSP2 was not (4). In addition, we have also observed that in double immunofluorescence

studies, incubating first with an anti-NSP5 serum strongly reduces the binding of the anti-NSP2, while the reverse does not (not shown). All together, these results suggested that in viroplasms NSP5 is more concentrated in an external layer while NSP2 would be located in a more internal position.

However, viroplasms appears to be dynamic structures, with an increment in size and a reduction in the number during time, suggesting a fusion process between them. The fact that the machinery of replication is in continuous change with an increase in size may indicate a requirement for the formation of DLPs. Within the reoviridae family, some viruses have also proteins with the characteristic of NSP2 and NSP5 to form VLS and localize in viroplasms. In reoviruses, σNS, the non-structural protein homologous to rotavirus NSP2, that has ssRNA binding activity and capacity to form higher order homo-oligomeric structures (92, 93, 118, 239) can also form inclusion body like structures when co-expressed with reovirus protein μNS, which although has not high homology to NSP5, may still play a similar role (20). In bluetongue virus (BTV) although it was described an analogy between NS2 and rotavirus NSP2, since they share NTPase activity, non-specific ssRNA binding and localization to inclusions bodies (83, 268), there are no reports describing its ability to form VLS when co-expressed with other viral protein. In spite of this, no analogy with rotavirus NSP5 has been reported for any protein of viruses of the Reoviridae family.

Mapping binding site with NSP2

Another goal of this thesis was to map in NSP5 the binding sites for NSP2. For this purpose, two different *in vivo* strategies were used: i) a two-hybrid system and ii) an *in vivo* binding assay in mammalian cells. Essentially, the two methods gave comparable results, indicating two main regions, the N-terminal and C-terminal parts of NSP5 (corresponding respectively to positions 1-33 and 131-198), as the main components required for the interaction. The results with the two-hybrid system suggested that a central region of 30 amino acids (region 3) was also important for NSP2 binding. Unfortunately, the corresponding deletion mutant Δ3 could not bein the assay used because of its transactivation activity.

However, in the immunoprecipitation assay while the role of regions 1 and 4T were clearly confirmed that of region 3 was not confirmed. In fact, this two terminal regions were able to confer to EGFP, when fused respectively at the N and C terminus, with the ability to localise to viroplasm in virus infected cells, and to form VLS in cells expressing only NSP2. The fact that the construct containing only the C-terminal fusion 4T from NSP5 was unable to form VLS suggested that indeed region 1 plays a crucial role in NSP2 binding, as it has been previously proposed(77).

NSP5 not only interacts with NSP2. It was also described interactions with VP1, VP6, NSP6 and recently, with VP2 (5, 21, 276). The consequences of these interactions, until now have not been resolved.

In this thesis, were clarified some aspects of NSP5 phosphorylation, its interaction with NSP2 and the requirements for localisation to viroplasms. On the light of recent findings in our laboratory, it is now clear that NSP5 is essential for the assembly of viroplasms, which in turns determines this ability of virus to replicate. It would be interesting to test some of the conclusion of the thesis in the context of the virus replication, but in the absence of the wtNSP5, which so far is not possible. In particular future studies should allow the determination of the link between NSP5 hyperphosphorylation and its role in viroplasms assembly and/or virus replication.

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