Scuola Internazionale Superiore di Studi Avanzati - Trieste

Dissecting the role of NSP5 in rotavirus replicative cycle by RNA interference

Thesis submitted for the Degree of
Doctor Philosophiae

Candidate
Michela Campagna

Supervisor
Dr. Oscar Burrone

Academic Year 2004/2005

SISSA – Via Beirut 2-4 – 34014 TRIESTE – ITALY

Dissecting the role of NSP5 in rotavirus replicative cycle by RNA interference

Thesis submitted for the Degree of

Doctor Philosophiae

Candidate
Michela Campagna

Supervisor
Dr. Oscar Burrone

Academic Year 2004/2005

Ai miei genitori
con affetto e riconoscenza

CONTENTS

ABSTRACT

Rotavirus genomes contain 11 double stranded RNA segments. Genome segment 11 encodes for the non structural protein NSP5 and, in some strains, also for the non structural protein NSP6. NSP5 is produced soon after viral infection and undergoes a complex post-translational hyperphosphorylation process that causes the generation of species characterised by reduced PAGE mobility. The use of deletion mutants demonstrated that NSP5 is able to autoregulating its own phosphorylation in a process that involves also the viral non structural protein NSP2 and cellular kinases. In particular, *in vitro* experiments had shown that casein kinase 1alpha (CK1α) was the kinase responsible of phosphorylating NSP5 on serine 67 and that this was the first step in the process of hyperphosporylation.

NSP5 localises in cytoplasmic viroplasms, structures where virus replication takes place. In viroplasms NSP5 has been shown to interact with the viral polymerase VP1, the structural protein VP2 and the non structural one NSP2.

In order to evaluate the role of NSP5 and its phosphorylation in the replicative cycle of rotavirus, we used the RNA interference approach.

In the first part of this thesis we targeted small interfering (si) RNAs to genome segment 11 mRNA of two different rotavirus strains and were able to block NSP5 production in a strain-specific manner. This allowed us to demonstrate that lack of NSP5 had a strong effect on the overall viral replicative cycle causing inhibition of viroplasm formation, decreased production of other structural and non structural viral proteins, block of the synthesis of viral genomic dsRNA and production of infectious particles.

In the second part of this thesis we wanted to evaluate if the results obtained on the hyperphosphorylation of NSP5 *in vitro* were confirmed *in vivo* and we decided to target a specific siRNA against CK1α. This siRNA showed to be very efficient in blocking CK1α production and allowed us to confirm in co-expression experiments that CK1α was the kinase responsible of phosphorylating NSP5 on serine 67. In addition we could show that in absence of CK1α also the phosphorylation of NSP5 in the context of viral infection was impaired. Lack of NSP5 hyperphosphorylation did not affect the interaction of NSP5 with the viroplasm resident proteins VP1 and NSP2 and was not involved in virus protein production. However, had a strong effect on the viroplasms formation and morphogenesis causing the formation of viroplasms with altered shape and dimension.

The results obtained demonstrate the essential role of NSP5 for the assembly of viroplasms, production of viral protein by de novo produced particle and virus replication. In addition the data presented confirm that also *in vivo* CK1α is the kinase responsible of phosphorylating NSP5 and suggest that its hyperphosphorylation is important for the correct assembly of viroplasms.

LIST OF ABBREVIATIONS

3' CS	3' consensus sequence
5' CS	5' consensus sequence
aa	amino acids
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ci	Curie
CK1	casein kinase 1
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	eukaryotic green fluorescent protein
eIF4GI	eukaryotic initiation factor 4GI)
ENS	enteric nervous system
ER	endoplasmic reticulum
FCS	foetal calf serum
GST	glutathione-S-transferase
GTP	guanosine triphosphate
hr	hour
HCV	hepatitis C virus
HIT	histidine triad
HIV-1	human immunodefiency virus-1
HRP	horse radish peroxidase
HSV-1	herpes simplex virus-1
ICAbs	intracellular antibodies
IPTG	isopropyl-β-D-thiogalactopyranoside
IRF-3	interferon regulatory factor 3
kDa	kilo Dalton
min	minute
NSP	nonstructural protein
N-terminal	amino-terminal
ORF	open reading frame
PABP	poly A binding protein
PAGE	polyacrilamide gel electrophoresis
PBS	phosphate buffer saline
PFU	plaque forming units
p.i.	post infect
PKC	protein kinase C
PKCI	protein kinase C interacting protein
RoXaN	rotavirus X protein associated NSP3
SDS	sodium dodecyl sulfate
SLP	single layered particle
TLP	three layered particle
UTR	untranslated region
VIB	viral inclusion body
VLP	virus like particle
VLS	viroplasm like structure
VP	viral structural protein

INTRODUCTION

In 1973, Bishop described a 70 nm virus particle using electron microscopy in duodenum of young children hospitalised for treatment of acute diarrhoea (20). Subsequently a series of studies from many countries reported the detection of the same virus in the faeces of young patients with diarrhoeal illness. It soon became apparent that this virus, named rotavirus for its wheel like appearance (latin *rota* = wheel), was the major etiological agent of acute infantile gastroenteritis infecting ~ 90% of children under the age of three and resulting in around 600 000 infants deaths each year (177).

1.1. VIRUS CLASSIFICATION

Rotavirus compose a genus within the family of *Reoviridae* and is characterised by series of common morphological and biochemical properties such as structure, genomic characteristics and replication process as described in table 1 (78).

TABLE 1. *General characteristics of rotaviruses*

Structure
65-to 75-nm Icosahedral particles
Triple-layered protein capsid
Nonenveloped (resistant to lipid solvents)
Capsid contains all enzymes for mRNA production
Genome
11 segments of dsRNA
Purified RNA segments are not infectious
Each RNA segment codes for at least one protein
RNA segments from different viruses reassort at high frequency during dual infections of cells
Replication
Cultivation facilitated by proteases
Cytoplasmic replication
Inclusion body formation
Unique morphogenesis involves transient enveloped particles
Virus released by cell lysis or by nonclassic vesicular transport in polarised epithelial cells

Rotaviruses are classified by three antigenic specificities: group, subgroup and serotype.

Group designation is given by the result of a series of tests based on the reactivity to monoclonal antibodies such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. All the epitopes that designate the group assignment are present on the structural proteins but VP6 is the predominant group antigen. Rotavirus comprises seven distinct groups (A to G) where groups A to C are found in both humans and animals whereas the remaining groups have been found up to now exclusively in animals. Viruses within each group are capable of recombination of the viral segments through a process of reassortment but this has been shown not to occur among viruses of different groups(253).

Subgroup designation is mediated by VP6 specificity and is an important epidemiological marker because most strains belong to either subgroup I or II. However the group and subgroup antigens do not induce the formation of neutralising antibodies although some VP6 specific polymeric immunoglobulins A (IgA) were shown to be protective *in vivo* (32, 217).

Rotaviruses are furthermore classified based on their serotype, defined as reactivity of viruses in plaque reduction neutralisation assays using hyper-immune serum (78). Neutralisation assays measure antibodies to both VP4 and VP7, the two proteins present on the outer shell of the virus, but the main reactivity is due to recognition of VP7, probably because it is the most abundant of the two proteins or because is more antigenic. However, since also VP4 causes the induction of neutralising antibodies, in the cases two viruses contain immunogenical different forms of VP4 but the same VP7 (since the two genomic segments can reassort independently), they can react differently in the neutralisation assays and not allow a clear identification of the serogroup. For this reason in designating a serotype two antigenic specificities are considered in a classification by a binary system, where the VP7 serotype is designated as G (because VP7 is a glycoprotein) and the VP4 serotype is designated as P (because VP4 is protease sensitive). Among group A rotaviruses at least 15 G serotypes and 23 P serotypes in humans have been identified, with G1 to G4 and P4 and P8 being the predominant ones; among these, G1P8 is the main strain, followed by G3P8, G2P4 and G4P8. However there have been reports of infections by unusual G serotypes and since the late 90's there has been an emergence of G9 serotype in many countries across all continents such as India, Bangladesh, United States, Brazil, Argentina, Italy, France, United Kingdom, Australia, Libya, Kenya, Cuba, Japan, Thailand and Taiwan (8, 10, 25, 26, 56, 113, 124, 175, 176, 204, 238, 262), and is now considered the fifth most important serotype in rotavirus infection (214, 249).

Thus the classification of rotaviruses comprises: a group, designated by roman capital letters (A-G), a subgroup, designated by Roman numerals, and two serotypes, G and P designated by Arabic numerals. It should however be noted that there is an increasing number of strains that are exceptions and for this reason, the distinctions are not always so clear cut, in particular for subgroups. Indeed there is an increasing number of viruses that cannot be classified in either subgroup or have both subgroup specificities.

1.2. STRUCTURE OF ROTAVIRUS

The first three dimensional structure of rotavirus was obtained by Prasad et al. in 1988 through the determination of the structure of particles of the viral strain SA11 with the use of cryo-electron microscopy and computer imaging (202). A following study on a different strain (RRV) (252) showed that the features were very similar to those of SA11 suggesting a similar structure.

Rotavirus is an icosahedral virus relatively large (~1000 Å) with a complex highly organised architecture composed by three concentric layers that surround two viral proteins involved in virus replication and transcription (VP1 and VP3) and the 11 dsRNA segments of the viral genome. The structure has a left handed T=13 icosahedral symmetry characterised by 132 aqueous channels and 60 surface spikes (202). The complete virions are also called Three Layered Particles (TLPs), particles where the outer layer is missing are also called Double Layered Particles (DLPs) and those that contain only the innermost layer are called Single Layered Particles (SLPs) or cores.

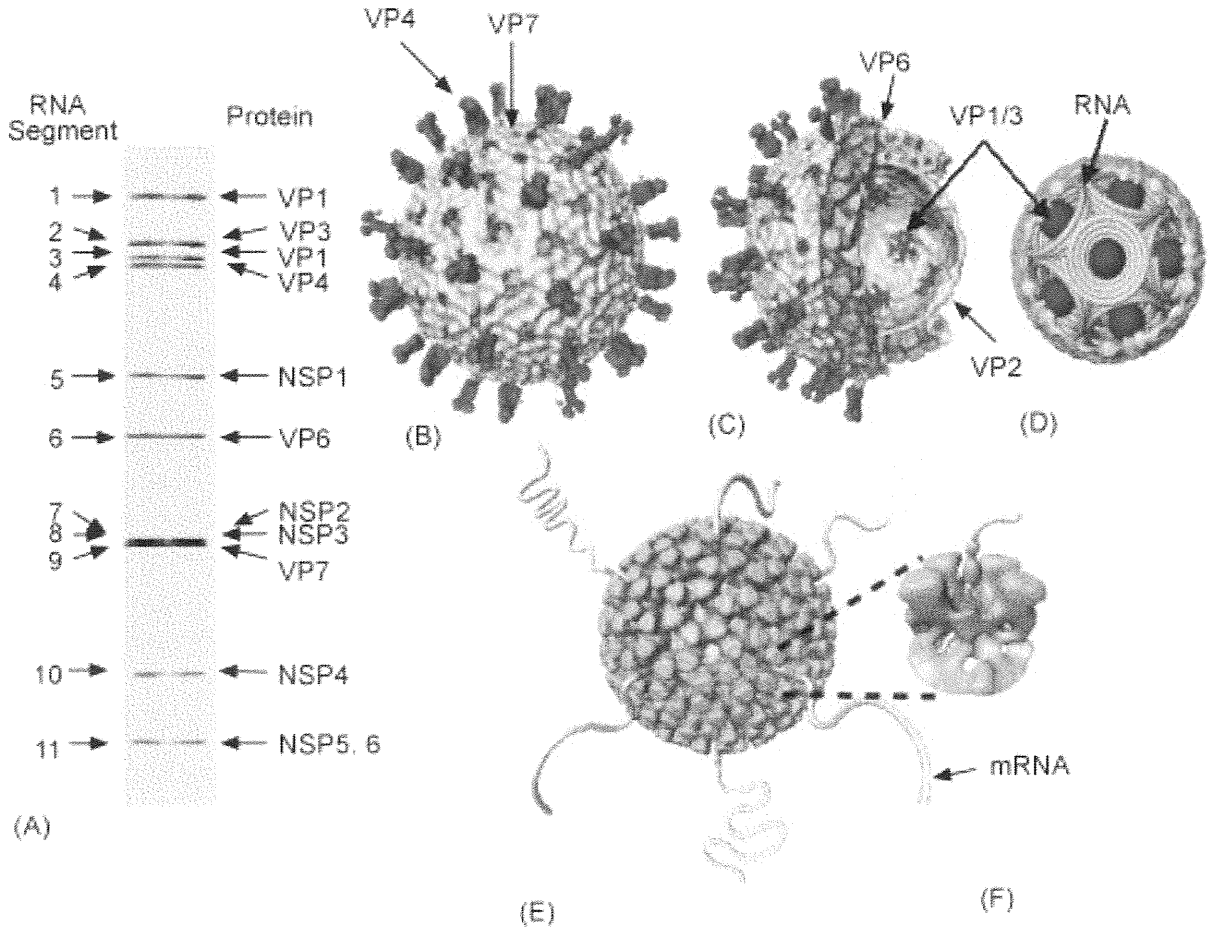


Figure 1: Architectural features of rotavirus. (A) PAGE gel showing, 11 dsRNA segments comprising the rotavirus genome. The gene segments are numbered on the left and the proteins they encode are indicated on the right. (B) Cryo-EM reconstruction of the rotavirus triple-layered particle. The spike proteins VP4 is colored in orange and the outermost VP7 layer in yellow. (C) A cutaway view of the rotavirus TLP showing the inner VP6 (blue) and VP2 (green) layers and the transcriptional enzymes (shown in red) anchored to the VP2 layer at the five-fold axes. (D) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as red balls) inside the VP2 layer in green. (E and F) Model from Cryo-EM reconstruction of transcribing DLPs. The endogenous transcription results in the simultaneous release of the transcribed mRNA from channels located at the five-fold vertex of the icosahedral DLP (114).

The aqueous channels

An important feature of rotavirus structure is the presence of 132 aqueous channels that are in register in the two outer shells crossing them to a deepness of about 140 Å. These holes are on average around 120Å distant from each other on the outer shell and 100 Å distant on the intermediate one (202). The channels have been classified in three types based on their localisation on the icosahedral structure. There are 12 type I channels that are localised on the icosahedral five fold axes, 60 type II channels on the 6 co-ordinated positions around the fivefold axis and 60 type III channels on the 6 co-ordinated positions around the threefold

axis. Type II and III channels are around 55Å wide on the outer face of the virus whereas channels I are narrower, 40Å in diameter. All the channels get narrower in the interior of the virus before widening again in proximity of the inner shell. The role of type I channels is allowing the exit of nascent mRNA synthesised in the inner core, as schematised in figure 1E, indeed the net charge in the walls of this type of channels is negative and this can facilitate the extrusion of the mRNA due to the electrostatic repulsion between the walls and the nucleic acid (157).

The outermost layer

The outermost layer is implicated in cell attachment and internalisation, therefore it is important in the first phases of virus infection. The protein mass, composed by the major outer layer protein VP7, is uniformly distributed and for this reason gives rise to a smooth outer surface from which 60 spikes protrude composed by the minor component protein of the outer shell VP4. VP7 molecules form trimers that interact in the T=13 icosahedral outer lattice so that there are 780 molecules of VP7 per virion (202).

VP4 spikes are localised near the edge of the type II channels and are dimeric so that each virion contains 120 copies of VP4 even if their status change during viral infection (67). The spikes protrude for about 100-120Å from the virus outer shell and are bilobed at the distal end (199, 252). About 30 kDa of VP4 protein are buried inside the virion surface and interact with the intermediate layer protein VP6 and are in close association with the walls of the type II channels made by VP6 (251).

The intermediate layer

The intermediate layer is composed by the structural protein VP6 that form 260 trimers and is the most abundant protein of the virion. Even though also this shell has a T=13 icosahedral symmetry, the protein distribution is not so uniform as in the outer shell, indeed the protein mass is mainly concentrated on the local and strict threefold axis giving the shell a typical bristle-like shape (202, 252). However there is a strong correlation between the two layers, indeed VP6 trimers lie under the VP7 ones and the 260 VP6 trimers are arranged in a way that the aqueous channels in the two layers are in register (202). Furthermore this layer contacts the inner one through mainly hydrophobic interaction between the basal region of VP6 and the inner core shell protein VP2 (38). Interestingly the most conserved regions of VP6 are those of interaction with the proteins of both the inner and outer layer (157).

The inner layer

This layer is composed by the structural protein VP2 (144). It consists of 120 molecules that are organised as 60 dimers of quasi equivalent monomers (designated A and B) forming a T=1 icosahedral lattice. This is the only layer protein that can form a closed shell when expressed in absence of the other viral proteins under physiological conditions (130) and for this reason it has been suggested to be the scaffold protein that allows the formation of the three layered particle.

VP2 dimers form a layer that is rather continuous and smooth on the exterior. The shell is interrupted by small pores that connect the inner core environment, where dsRNA is present, with the outside. Type I and Type II channels terminate at this shell whereas type III channels continue beyond this layer (200).

Subcore and organisation of the genome

VP2 is known to have the capability of binding through its N terminal region the replicase/transcriptase complex formed by VP1 and VP3 (138, 257). By comparative cryo-electron microscopy analysis of DLPs and recombinant virus like particles, it has been shown that a small portion of VP2 protrudes inward at the fivefold axis with a pentagonal shape that anchors the replicase/transcriptase complex (201). This localisation is consistent with the extrusion of nascent mRNA through the type I channels located at the fivefold axis (136).

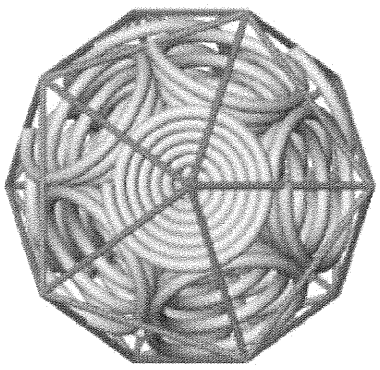


Figure 2: Schematic representation of the structural organisation of the genome icosahedrically ordered. Each dsRNA forms an inverted cone at the 5-fold vertex Modified from (186).

The precise organisation of the genome inside the innermost shell has still to be completely elucidated but it has been shown that the genome forms concentric layers (figure 2), generally separated by a distance of 28-30Å (186), that surround the fivefold axis encircling the VP1/VP3 complex (201). A plausible model for RNA arrangement is the one of bluetongue virus (98) where each dsRNA molecule is bent in a way that resembles an inverted coil around the transcriptase complex at the fivefold vertex of the icosahedral structure (186).

This type of organisation also explains why up to now no member of the reovirus family has been found to have more than 12 genomic segments. It is however interesting also to notice that most members of this family have 10 or 11 segments and not 12.

1.3 STRUCTURAL PROTEINS

1.3.1. VP1

VP1 is a basic protein produced by the first segment of rotavirus genome. It represents the viral RNA polymerase and functions both as transcriptase (mRNA synthesis) using as a template the minus strand of the dsRNA and as replicase (minus strand synthesis) using as a template the plus strand RNA. Consistent with its role in replication VP1 together with VP2 is the minimal requisite to obtain replication of viral mRNAs in an *in vitro* system (44, 258). In addition other indirect evidences of the fact that VP1 is the viral polymerase are the fact that VP1 shares four conserved regions of homology with RNA polymerases of other RNA viruses (48, 85) and that the use of the nucleotide analogue azido-ATP was able to block transcription through its binding to VP1 (239). It has been also shown that VP1 has both specific affinity for RNA (i.e. interacts with the 3' end of gene 8 mRNA in absence of other viral proteins) and non specific affinity for RNA a feature expected for a polymerase (178, 180).

1.3.2. VP2

VP2 is a protein of 882 amino acids, product of the second segment of rotavirus genome.

The protein is characterised by an N terminal RNA binding domain of 132 residues that contains several motives that have been predicted could bind RNA (129). Through this region VP2 is able to interact aspecifically both with ss and dsRNA, although the affinity for ssRNA is greater that the one for dsRNA (29). It has been shown that the deletion of just the first 26 residues abolishes the RNA binding activity suggesting that these first amino acids either play an important role by themselves or are necessary for the proper conformation of the N terminal region (129). The amino terminus region of VP2 has been shown to localise around

the five fold axis of the icosahedral structure and to contact RNA several times particularly around each vertex (138). These multiple interactions may help induce the bending of the dsRNA molecule inside the core and impose a precise order in this bending that is necessary for the subsequent coordinated actions of the transcriptional process (133). The protein contains also two leucine zippers between amino acids 536 and 686 that could be involved in the oligomerisation of the protein into cores (179).

VP2 interacts both with VP1 and VP3 through the N terminal domain as demonstrated by the fact that an N truncated form of the protein lacks the ability of incorporating the transcription/replication enzymes VP1 and VP3 (257). Furthermore VP2 is known to interact with VP6 and co-expression of the two proteins in a baculovirus system in absence of the other viral proteins causes the formation of VP2/6 double layered Virus Like Particles (VLPs) (130). In addition VP2 has been shown to interact with the non structural protein NSP5 and this interaction as well as the one with VP6 does not involve the N terminus region of VP2 (18). Interestingly the studies of co-expression of VP2 with NSP5 in baculovirus have shown that the localisation of VP2 inside the cell is strongly influenced by the presence of NSP5 and VP6; indeed expression of an amino terminal deletion mutant of VP2 fused with GFP causes the formation of evenly distributed small fluorescent inclusions, whereas the co-expression of NSP5 results in the formation of fewer but much bigger inclusions containing both VP2 and NSP5 and this process is hindered by the presence of VP6 (18).

VP2 is able to self assemble into a stable capsid when expressed in the absence of the other viral proteins (130) and this characteristic is independent of the N terminal region (138). VP2 has a crucial role in the assembly of the virion to function as scaffold protein for the proper assembly of the genomic dsRNA and encapsidation of VP1 and VP3. This structure is the minimal necessary for the replication of the virus in an *in vitro* system as it has been shown through studies using temperature sensitive mutants (155) and baculovirus co-expression systems (44).

1.3.3. VP3

VP3 is the product of the third segment of rotavirus genome and is probably the less characterised of all the rotaviral proteins. It is a basic protein with unspecific affinity for ssRNA but not for dsRNA (180). VP3 is one of the minor components of the virion core (144),

a component of early replication intermediates and, together with VP1, seems to be the first protein to interact with RNA during packaging and genome replication (87). As previously mentioned VP3 is able to interact with VP2 in a region where also VP1 and ssRNA are known to bind forming the complex responsible of the transcription of viral mRNAs (129, 257). The protein is known to interact with VP6 and have been suggested that this interaction can be essential for transcription (213). Several studies have demonstrated that VP3 is the viral methyl-transferase and guanyly-transferase responsible for capping of the viral mRNAs (42, 145, 191).

1.3.4. VP4

VP4 is the product of the fourth segment of rotavirus genome. It is a non glycosylated outer capsid protein of 776 amino acids with a molecular weight of around 88kDa. Increasing number of studies highlight the importance of VP4 in the biology of rotaviruses. VP4 has been shown to be the hemagglutination protein of the virus; indeed attaches to sialic acid containing cell surface components (81). Furthermore VP4 is a determinant of virulence (82, 96) and antibodies against VP4 are neutralising (108, 174).

VP4 is localised in the outer shell of the virion where it is present in 120 copies and forms 60 dimeric spikes of 10-12nm (199, 252). It can be subdivided into an amino-terminal head, a body, a stalk and a C terminal foot that extensively interacts with the protein VP6 of the intermediate virus layer anchoring VP4 to the virus (220, 251). VP4 is also making close contacts with VP7 (the other viral protein present in the outer layer of the virus) (251).

Trypsin treatment of VP4 is required for virus infectivity. Indeed rotaviruses, that are not proteolitically activated, do not infect cells (120). VP4 is cleaved by trypsin at arginines 231, 241 or 248 (9, 146) and comparison of trypsinized and non-trypsinized rotavirus particles have shown that trypsin cleavage stabilises the spike. In non-trypsinized particles the VP4 spikes are disordered suggesting a flexible conformation whereas trypsin treatment results in a better definition of the VP4 spike corresponding to a better ordered conformation (53). Trypsin digestion causes the cleavage of VP4 into two proteins that remain both associated with the virion: VP8* (~28kDa) and VP5* (~60kDa).

VP8* forms the globular head of the spike and contains the viral sialic acid binding and hemagglutination motif (81). Structural analysis have demonstrated that the VP8* core is a

single compact and rigid globular domain composed by two β sheets respectively of five and six β strands flanked by two small α helices (65, 128). Between the two sheets is present a cleft filled of hydrophobic side chains and in this region is predicted to be the sialic acid binding site. The fold of the VP8* core resembles that of galectin, a family of sugar binding proteins, even though the two proteins do not share significant sequence similarity and no clear function correlation up to now has been found (68). However it has been suggested it could have a role in virus exit (215) (as described in § 1.6.5.).

The majority of VP4 is composed by the other trypsin cleavage product VP5*. Many of the functions of VP4 have been shown to localise in the VP5* region. It can permeabilise membranes (61) and contains a hydrophobic putative internal fusion domain homologous to the fusion domain of the E1 protein of Sindbis virus and Semliki Forest virus (153), that was shown to be responsible for the permeabilising capability of VP5* (69). Due to this property it has been suggested VP5* to have a role in the process of virus entry (61, 69, 91).

Since VP4 forms the spikes of the virus and mediate the first contact between the virus and the target cell, many studies have searched for cellular proteins that could interact with VP5*. These studies have demonstrated that VP5* interacts with integrin α2β1 (51, 100, 106). Integrins are a family of cell surface receptors important for adhesion between cells and between cellular membrane and the extracellular matrix, that have a role in the regulation of cell differentiation, proliferation and survival (112).

Apart from integrins also the Heat Shock Cognate Protein 70 (Hsc70) has been shown to interact with VP5* (102, 255). This is a constitutive member of the heat shock-induced protein family of molecular chaperones that functions in normal cellular physiology.

Also the structure of a VP5* fragment corresponding to the globular domain of VP5* and a part of the stalk, but missing all the C terminal region of the foot interacting with VP6, has been crystallised. This have shed new light on the rearrangements that occur on viral outer shell upon trypsin treatment and cell binding. The structure is a well ordered homotrimer that resembles a folded umbrella. Is composed by an N-terminal globular domain (the shade of the umbrella) and by a C-terminal triple α-helical coiled coil (the post of the umbrella). Each globular domain is an antiparallel β sandwich composed by eight strands and interestingly one of the hairpins that connects two beta strands and protrudes towards the solvent comprises the peptide (DGE) responsible for the recognition of the α2β1 integrin. Furthermore the loops projecting at the bottom end of the globular domain create a hydrophobic region that could be involved in membrane penetration, indeed there is some similarity between this region and the

fusion loop of alphavirus (67). The trimeric structure of VP5*CT is in contrast with previous reports that had shown VP4 to be a dimer (199) and have suggested that VP4 is an highly flexible protein that can be present into three different conformational statuses during cell infection. Recent studies have shown that at high pH the conformation of the spikes is clearly trilobed supporting the idea that the protein can be present in different conformations (185). The role of these changes could be masking some epitopes during cell entry and show them in a sequential pattern in order to interact with different receptors on the cell membrane and allow rotavirus binding and internalisation. In this kind of model the not trypsinized virion presents a very flexible VP4 stalk, upon trypsin cleavage VP4 gets dimeric and rigid and exposes the VP8* and VP5* epitopes for cell binding whereas the third molecule of VP4 continues to be flexible. A third unknown event triggers the passage to a third conformation in which the VP8* protein is lost and the three VP5* trimerise with the exposure of the hydrophobic region with permeabilising characteristics (67).

During virus infection neo synthesised VP4 localises in the cell in the space between the periphery of viroplasms and the outside of ER (188), furthermore VP4 has been shown to interact, together with VP7, with NSP4 (152). In fact NSP4 contains in its C-terminal tail a binding sequence for VP4 (12). However no clear evidence of the presence of VP4 in the immature virion inside the ER has been found (92, 215). Further studies have shown that VP4 is also localised at the plasma membrane early after infection (171) and that the protein associates with rafts, membrane lipid microdomains enriched in cholesterol, sphingolipids and particular proteins that play a key role in numerous cellular functions such as vesicular trafficking. This has suggested that the final steps of viral morphogenesis occur at the plasma membrane level in lipid rafts (55, 171, 215). This kind of interpretation is also in agreement with the results obtained through the use of RNA interference (RNAi) (described in § 1.9.). The lack of VP4 production through RNAi (58) caused the formation of rotaviral spikeless TLPs efficiently suggesting the assembly of VP7 to be precedent and independent of the one of VP4 (58).

Through the use of the two hybrid system it has been shown VP5* to interact with two proteins involved in vesicular trafficking: rab5, a protein of the family of Ras- related monomeric GTP binding proteins involved in the control of fusion events, and PRA1 (Prenylated Rab Acceptor 1), a protein that localises in Golgi membranes and rafts and plays an important role in recruiting and keeping rab5 in a membrane bound form (1, 156). In particular it has been shown that the interaction of these two proteins occurs with free

cytosolic VP4 and it has been suggested that these interactions could have a role in the last stages of viral morphogenesis (75).

VP4 and VP8* have also been shown to interact inside the cell with TRAF2, a member of a family of adapter proteins that have the role of transducing tumour necrosis factor signals to intracellular partners among which NFκB, suggesting a possible interference of VP4 in the cellular signalling pathway (132).

1.3.5. VP6

VP6 is encoded by genome segment six of rotavirus genome. It is the most abundant protein of the virus and is the major component of the virions exerting the crucial role of connecting the outer layer of the virion with the inner one and in this way acting as a linker between the functions of the other two layers (virus entry and genome packaging). Furthermore the protein has been involved in the process of transcription since in its absence this process does not take place, even though it is not yet clear if the protein has just a structural role or is more directly involved in the transcription process (19, 38).

VP6 is known to interact with VP2 and the co-expression of the two proteins in insect and mammalian cells causes the formation of double layered virus like particles (Dl-VLPs) (94, 130). Furthermore the use of an insect system allowed to show that when VP2 and VP6 were expressed together with VP4 and VP7 TLPs were formed and that co-expression of VP2/6/4 or VP2/6/7 caused as well the formation of particles highlighting the importance of VP6 for the self assembly of viral particles (52).

VP6 is a trimer (97, 211) and when expressed alone in absence of other viral proteins can form tubular or spherical structures formed by dimers of trimers of VP6 (140, 206). It has been shown that the type of structure formed depends upon the pH: at pH 3.5-5.5 spherical particles were formed while in the range 5.5-7 large tubes were formed and with a pH above 7 small tubular structures were formed suggesting that the protonation status of the protein is important for its assembly (140, 237).

X ray crystallography have shown VP6 trimer to be an elongated molecule resembling a tower 95Å long with a base with a roughly triangular shape of ~60Å that contacts the inner layer and a head roughly exagonal with a diameter of 45Å that contacts the outer layer (157). At the centre of the trimer a Zn^{2+} ion coordinates to an His in each monomer (His 153) and

contributes to stabilise the trimer. Mutations of this zinc binding histidines into serines prevent Zn^{2+} coordination and render the trimer more susceptible to cleavage from proteases but has no effect on the interaction with VP2 or on the transcription capability of the DLP (77). Each VP6 monomer can be divided in two distinct domains that have been called domain B and H. Domain B is at the base of the structure and is the region of interaction with VP2. It consists of a bundle of 8 α helices derived by the first 150aa and by the residues 335-397 of the VP6 sequence. The domain is also characterised by a β hairpin highly conserved that extends laterally at the base of the molecule directed towards channels I formed by VP6 at the icosahedral fivefold axis. It has been suggested that the negative charge present in this channel, to which the β-hairpin contributes, could have a role in the extrusion of the neo-synthesised mRNA from the DLP. Domain H that comprises the rest of VP6 sequence folds into a β-sandwich to produce a β-roll (157). Hydrophobic interactions in the H domain are mainly responsible for the trimerisation of VP6. Mutants in which this region is deleted cannot trimerise whereas mutants that are lacking regions of domain B have still the capability of trimerising (4).

In infected cells VP6 localises in the region at the periphery of viroplasms and in close proximity of the ER (188). It has also been shown that NSP4 is able of interacting through its cytoplasmic tail with VP6 and this could have a role in the assembly of the intermediate layer or in the budding of DLPs from the viroplasms to the ER (11, 12, 147).

1.3.6. VP7

VP7 is encoded by the seventh segment of rotavirus genome. It is the viral glycoprotein that forms, together with VP4, the outer layer of rotavirus virion.

Both in infected and transfected cells VP7 localises in the ER membrane where it is assembled in the maturing virion during its transit. However the conformation of the protein in the ER is not known but it is assumed to have a luminal orientation (118) and to interact with the viral non structural protein NSP4 and with VP4 (152).

VP7 open reading frame codes for a protein of 326 residues that is characterised by high mannose carbohydrate glycosylation sites that are supposed to be of little importance for virus assembly since rotavirus strains with none or only one glycosylation residue assemble and produce fully infectious particles (119, 188). In addition VP7 is characterised by having

several cysteines (eight in the group A) that are highly conserved and that form disulphide bonds important for the correct folding of VP7 in a process that requires ATP (164).The N terminus of VP7 is characterised by two hydrophobic domains each of them preceded by a methionine. The first hydrophobic domain is usually not translated with translation starting from the second methionine. In vitro translation and in vivo transfection experiments have shown that the two domains cause the targeting of VP7 to the ER and the second hydrophobic domain is considered the main responsible for this targeting. Upon localisation in the ER the hydrophobic region is cleaved regardless of whether both domains are present or only one resulting in a mature VP7 with a glutamine at the N terminus (198, 225, 247). After the cleavage of the two hydrophobic domains the N terminal region of VP7 is necessary and sufficient for its retention in the ER, in particular residues Ile-9, Thr-10 and Gly-11 of the mature protein are essential (151) however this region does not confer membrane binding properties to VP7 and so far the mechanism of membrane binding of the protein has not been elucidated. VP7 contains a highly conserved C terminal region that is temporarily embedded in the membrane and protrudes in the cytoplasm that had been suggested to have a role in the membrane binding capability of VP7 (47), but it has been shown that this region is not the sole responsible of VP7 membrane binding (47).

One important characteristic of VP7 is its capability of interacting with Ca^{2+} and two prolines, localised one at the N terminus and the other one at the C terminus of VP7, are important for Ca^{2+} binding (86). It has been shown that in presence of Ca^{2+} VP7 is able to trimerise and that consequently a decrease in Ca^{2+} concentration dissociates the trimers, a result that could be connected with the virus uncoating (loss of the outer shell) that occurs upon virus entry in the cell (66). In agreement with this result it has been shown that the capability of losing the external viral layer during virus entry, due to the decrease in calcium concentration between the extracellular compartment and the intracellular one, segregates with the genomic segment coding for VP7 (208). In absence of Ca^{2+} VP7 undergoes conformational changes that alter its capability of being recognised by monoclonal antibodies (64). In addition Ca^{2+} interaction is important for VP7 interaction with VP4 and NSP4 in the hetero-oligomeric complex that is important for the assembly of the outer layer (152, 197).

VP7 have been also implicated in the first steps of cell binding since interacts with integrins $\alpha v\beta 3$, that binds the VP7 sequence CNP (256), and the integrin $\alpha x\beta 2$, that binds the sequence GPRP (100). Furthermore VP7 has been shown to permeabilise membranes when

solubilised from VPL2/6/7 and trypsinised suggesting a role, similar to the one of VP4, in the intracellular entry of the virus (37).

1.4. NON STRUCTURAL PROTEINS

1.4.1. NSP1

NSP1 (previously known as NS53) is encoded by genome segment 5. It has a molecular weight of ~54kDa and is the least conserved protein encoded by the rotavirus genome (165). The only conserved region of the protein is the N terminal one, which is characterised by the presence of eight cysteines and two histidines residues that are part of a zinc finger domain; this region has specificity for all the 11 viral mRNAs recognising an element localized on the 5' region of the mRNAs (30, 109)

NSP1 is localised throughout the cytoplasm of infected cells and has been found to be associated to the cytoskeleton (110). The protein is not required for rotavirus replication, indeed rearranged strains in which NSP1 is truncated are able of replicating to the same level of strains with a functional NSP1. Furthermore experiments in which the expression of the protein was blocked through the use of RNA interference showed no difference in the production of other viral proteins, in the replication of the virus and in the formation of viroplasms, showing NSP1 not to be essential for virus replication (221). However viruses not expressing NSP1 show a small to minute plaque phenotype suggesting the protein to be involved in modulating host response to the virus. Indeed NSP1 has been demonstrated through yeast two hybrid system to interact with the interferon regulatory factor 3 (IRF-3) (99). IRF-3 is a ubiquitously expressed 55kDa protein that accumulates as inactive monomer in the cytoplasm (13). Upon viral infection a series of signals such as virus entry, production of dsRNA and expression of viral proteins can induce the phosphorylation of IRF-3 by cellular kinases that causes a conformational modification of the protein and its subsequent dimerisation and translocation to the nucleus where IRF-3 interacts with specific promoters and induces the expression of IFN-α and of IFN-β. The IFNs are able to signal the activation of an antiviral response in the neighbouring cells blocking in this way the virus spread (125). It has been demonstrated that upon rotavirus infection NSP1 has the capability of directly

binding to IRF-3 and causing its degradation through a proteasome dependent pathway and of inhibiting its dimerisation and consequent translocation to the nucleus. In this way NSP1 is able of subvert the innate cellular response to viral infection (15). It is also interesting to notice that the high degree of variability of NSP1 in the various rotavirus strains may be due to the necessity of the protein to be able to specifically block the host innate response in a species-specific manner. This may in part explain why rotavirus infection is often asymptomatic in non homologous animal models (70).

1.4.2. NSP2

NSP2 (previously called NS35) is a basic, conserved 35kDa protein coded by genome segment eight. Sedimentation studies of NSP2 derived by infected cells or produced as recombinant protein have shown that the protein self assembles into highly stable octamers (216).

Octamers are the functional form of the protein. Temperature sensitive mutants of NSP2 (tsE), characterised by the failure of NSP2 to form at the non permissive temperature the octameric unit, are defective in NTPase and helix destabilising activity (205, 232).

Structural studies of NSP2 at a resolution of 2.6 Å have shown that the octamer has a donought shape with a 35Å central hole, formed by the head to head stacking of two tetramers (115). Each NSP2 monomer is characterised by two distinct domains separated by a 10 residues loop. The N terminal domain is mainly alpha helical and has two pairs of anti-parallel beta strands towards the N terminus. The domain can be further subdivided into two sub-domains connected by a loop that contains three highly conserved prolines. This loop contributes to the formation of a groove that runs diagonally in the octamer and is supposed to be the site of localisation of viral RNA. The C-terminus domain of NSP2 is characterised by antiparallel beta-sheets surrounded by a central alpha-helix (115). Whereas the use of the DALI server did not show any statistically significant similarity with known domains in the N terminal part of the protein, the C terminal domain has a significant homology with the structure of the protein kinase C interacting protein (PKCI), a member of the Histidine Triad (HIT) family of nucleotidil hydrolases (142). Even though the proteins do not have sequence similarities and the characteristic HIT motif is not conserved in NSP2, it has been shown that the protein retains His 225 and other nearby residues predicted to be important for the NTPase

activity typical of the members of the HIT family (34). The two distinct domains of NSP2 are separated by a 25Å deep cleft that has been suggested to be the site of NTP hydrolysis (34, 115). NTP hydrolysis is accompanied by auto-phosphorylation of the protein (229), but the phosphorylated species is not stable. Indeed, if the hydrolysis activity of the protein is inhibited, a rapid dephosphorylation occurs, suggesting that the formation of a transient phosphorylated intermediate is followed by a dephosphorylation step in which a second His, different form His 225, seems to be involved (34). The rate of dephosphorylation is however slower than the rate of phosphorylation of NSP2 accounting for the detection of the phosphorylated species of NSP2 in the NTPase assays.

A series of biochemical studies have shown NSP2 to have a non specific affinity for ssRNA (122, 229). The binding of ssRNA occurs in a cooperative fashion in which various NSP2 octamers bind a unique molecule of mRNA (230). Furthermore biochemical studies, in agreement with structural ones, have shown that NSP2, has a Mg^{2+} dependent NTPase activity, can catalyse the hydrolysis of all four nucleosides triphosphate (229) and has a nucleotide-Mg^{2+}- independent helix destabilising activity (230).It has been demonstrated that the presence of NTPs and Mg^{2+} have the capability of stabilising the octamer structure whereas the only presence of Mg^{2+} has the opposite effect, causing the dissociation of the octamer in two tetramers (216). It has been suggested that these activities have the role of removing duplexes formed by the viral mRNA that would block the process of packaging.

NSP2 is known to localise in cytoplasmic viroplasms where genome replication and packaging take place. It has been demonstrated that co-expression of NSP2 with NSP5 (the other non structural protein that localises in viroplasms) in absence of the other viral proteins causes the formation of empty structures that resemble viroplasms and are called Viroplasm Like Structures (VLS) (80). In addition to NSP5 and NSP2, in viroplasms localise VP1, VP2, VP3 and viral mRNAs, that are the components of core replication intermediates, the viral structure that retains replicase activity (mRNA→dsRNA) (87, 181), suggesting a role for NSP2 in the virus replication. Indeed, NSP2 has been shown to be tightly associated with the viral replicase machinery (7), to compete with VP2 for the binding to the viral mRNAs and to inhibit dsRNA production by interfering with the formation of the initiation complex for minus strand synthesis (244). Furthermore blocking NSP2 production by RNA interference causes a complete inhibition in viroplasms formation, production of viral proteins and viral replication (221).

All these data taken together suggest that the NSP2 octamer is strongly involved in the mRNA unwinding and packaging, but how the octamer functions in facilitating these tasks is still not understood.

1.4.3. NSP3

NSP3 (previously called NS34) is a slightly acidic protein of 35kDa coded by genomic segment nine. The protein is spread through the cytoplasm of infected cells in a filamentous manner and for this reason an association with the cytoskeleton has been suggested (158).

The two hybrid system used to screen a cDNA library of mRNAs coming from the monkey kidney cell line CV1, that is susceptible to rotavirus infection, has allowed to identify two cellular interactors for NSP3. The first interactor found is the protein eIF4GI (eukaryotic Initiation Factor 4GI) (190). This is a scaffold protein that has the role of assembling the cellular translational complex composed by the proteins eIF4E (cap-binding protein), eIF4A (helicase), PABP (Poli-A Binding Protein) and eIF3. All this complex, in which the mRNA is circularised, is then loaded by the subunit 40S of the ribosome that begins the scanning of the 5' UTR in order to find the first methionine where to begin translation. The fact that NSP3 interacts with one of the proteins of this complex has suggested that it is involved in the translation of the viral mRNAs and in the shut off of cellular protein synthesis that occurs upon viral infection (190). In particular it has been demonstrated that NSP3 is able to interact with eIF4GI in the same region where PABP binds competing with it for the binding (190). PABP is the protein that binds to the poly-A tail of cellular mRNAs and is involved in mRNA stability, poly-adenylation and translation. Even though viral mRNAs are capped at the 5' UTR with a 7-methyl-G (5')ppp(5')N as cellular mRNAs, differ from these because they lack the poly-A tail. The role of NSP3 seems to be sequestering of the initiation complex of translation allowing only the recruitment of viral mRNAs.

NSP3 recognises and specifically binds to the 3' end of rotavirus mRNAs and this interaction protects the 3' end from RNase digestion (193). Subsequently it has been demonstrated that the terminal 4 nucleotides (GACC), that are common to all viral mRNA segments, are the shortest target for the specific interaction of NSP3. Indeed, mutations in this sequence affected NSP3 affinity for RNA (194). Interestingly NSP3 is able of recognising only the mRNAs coming from the same serogroup showing a very precise interaction among

the protein and its target mRNAs (194); this characteristic could account at least in part for the inability of rotaviruses coming from different serogroups of reassorting among each other.

Structural studies have helped to understand how the interaction among NSP3, eIF4GI and mRNAs occur. NSP3, whose active form is a dimer, consists of two separable domains. A N-terminal part binds to the rotaviral mRNA 3' consensus sequence (193); this domain forms an highly asymmetric heart-shaped dimer that contains an inner cleft where the consensus sequence is positioned. The RNA is completely buried in a highly basic tunnel where the tetranucleotide is recognised by a network of hydrogen bonds, van der Waals contacts and salt bridges. The tunnel is closed at one end thereby excluding the possibility that internal sequences can be recognised. NSP3 has a high affinity for RNA binding and a slow dissociation rate that are consistent with its role of causing the selective translation of viral mRNAs and protecting the 3' from the action of exonucleases (62). The C domain of NSP3 is instead involved with the recognition of eIF4GI, it is a symmetric homodimer composed by three pairs of alpha helices that form a rod shaped structure containing two binding sites for eIF4GI (101).

Interestingly the use of two hybrid system has allowed the identification of another NSP3 interacting protein that has been called RoXaN (Rotavirus X protein associated with NSP3). This protein has been shown to interact with the dimerisation domain of NSP3 and to form a ternary complex together with eIF4GI during rotavirus infection, however its role has not yet been understood (245).

1.4.4. NSP4

NSP4 (previously called NS28) is a glycosylated protein of ~28kDa coded by genomic segment ten. Concomitantly with translation, NSP4 is glycosylated to a 29kDa species and subsequently the glycosylation is processed with the formation of the definitive 28kDa protein that contains two high-mannose N-linked oligosaccharide residues (118).

NSP4 has been extensively studied both because it plays a pivotal role on the morphogenesis of the virus and because it has been shown to be an enterotoxin responsible of many symptoms of rotavirus infection.

The protein is localised in the membrane of the ER and can be subdivided in three N terminus hydrophobic domains, a coiled coil domain and a C terminus domain. The first short

hydrophobic N terminus domain is exposed in the lumen of the ER and is N mannose glycosylated. The role of this glycosylation has not yet been understood, although has been suggested to be critical for the assembly of rotavirus virions (59, 187). However subsequent studies have challenged this view (163). The second hydrophobic domain is a 25 residues transmembrane region that traverses the ER bilayer. Most of the protein is thus cytoplasmic. The coiled coil domain was shown to be necessary for oligomerisation of the protein and causes the protein to form dimers and tetramers stabilised by Ca^{2+} (28, 152, 233). This is an interesting characteristic since Ca^{2+} mobilisation by NSP4 is considered to be one of the ways in which the protein exerts its enterotoxic effects. The C terminus region of the protein contains the binding sites for other two viral proteins: VP6 binds to the 20 extreme C terminal residues of NSP4 (173, 233) and, in close proximity with it, there is the binding site for VP4 (12). The use of deletion mutants have demonstrated that in the absence of the residues that are necessary for VP6 binding, the binding of VP4 increases suggesting that the C-terminal binding domain for VP6 could partially obstruct the VP4 binding site. NSP4 has been shown to hetero-oligomerise with VP4 and VP7 (the two proteins that form the external layer of the mature virion) (152). These interactions could have a role in the morphogenesis of viral particles. It has been indeed demonstrated that NSP4 plays an important role in the last steps of virus morphogenesis: after the assembly of the cores into viroplasms these are known to acquire the second layer of VP6 forming DLPs and budding into the ER. This process is mediated by NSP4, indeed in absence of NSP4 no budding occurs (147).

Another study have also identified in the last 54 residues at the C terminus of NSP4 a microtubule-binding domain: this sequence, that does not share significant homology with other microtubule associated proteins, seems to have a role in binding microtubules and preventing the trafficking between ER and Golgi (250).

Interesting information about the role of NSP4 in viral morphogenesis comes from the utilisation of RNA interference (147). This study has demonstrated that in absence of NSP4 the accumulation and distribution of other viral proteins are altered. In particular it could be observed a very strong decrease in the synthesis of VP2, NSP2, VP7, VP4 and NSP5, whereas the levels of VP1 and VP6 were unaltered and, surprisingly, the production of NSP3 was increased almost twofold. As previously stated, NSP4 is able of mobilising Ca^{2+} inside the cell and one possible explanation for the altered production of the viral proteins could be the necessity of an increased Ca^{2+} concentration for their stability or production. Furthermore immunofluorescence studies showed that also the cellular distribution of viral proteins was

altered: VP6, that usually has a peri-viroplasmic location, was forming filaments that appeared to extend to the periphery of the cell, VP2 was seen to distribute homogeneously on the cell instead of being localised in viroplasms, VP4 was not anymore forming peri-nuclear structures close to the viroplasms but it was forming filamentous structures and, similarly, VP7 had a more diffuse pattern of distribution. The distribution of NSP3, NSP5 and NSP2 instead was not changed. Another characteristic of the lack of NSP4 was the absence of both DLPs and TLPs where the lack of DLPs can be ascribed to the necessity of NSP4 for the assembly of VP6, as suggested by the fact that the two proteins interact (147). However since NSP4 is not needed for the formation of VLPs such as VLp2/6, VLP2/6/7 or VLP2/6/4 in cotransfection experiments (52, 94, 130) its role in the process of DLP formation has still to be elucidated.

As previously mentioned NSP4 was shown to be the first enterotoxin of viral origin. Indeed purified NSP4 or a peptide $NSP4_{114-135}$ are able of inducing diarrhoea in young mice suggesting a strong link between this protein and rotavirus pathogenesis (as described in § 1.7.).

1.4.5. NSP5

NSP5 (previously known as NS26) is the product of the longer ORF of genome segment 11 (159). It is a protein of 196-198 amino acids (depending on the virus strain) with a high content of serine (21%) and threonine (4.5%). The protein is synthesised soon after viral infection (2-4 hrs) and is continuously produced.

NSP5 was originally described to have a molecular mass of 26kDa on SDS-PAGE. Further studies demonstrated that the 26kDa form is a precursor of higher molecular weight forms. In virus infected cells NSP5 appears as two major bands of 26 and 28kDa and as a series of higher bands whose apparent molecular weights span from 30 to 34kDa (figure 3 lanes 1 or 2).

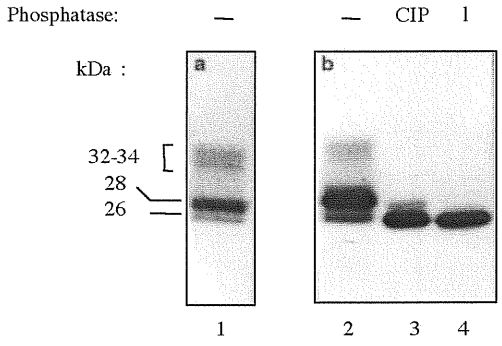


Figure 3: SDS-PAGE analysis of NSP5. Lane 1: immnunoblot analysis of extracts of SA11-infected MA104 cells (4h post-infection) reacted with anti-NSP5 serum. Lanes: 2-4: immunoprecipitation of NSP5 from virus-infected cells labelled *in vivo* with [^{35}S]-methionine and treated with phosphatases as indicated. From (6)

Two types of post translational modifications have been shown to occur in NSP5: O-glycosylation (95) and phosphorylation (6, 22, 195).

Cytoplasmic O-glycosylation occurs by addition of O-linked monosaccaride residues of N-acethylglucosamine to serines or threonine of cytoplasmic or nuclear proteins. Labelling of virus infected cells with [1,6-^{3}H]glucosamine showed that both the 26kDa and the and 28kDa forms of NSP5 are O-glycosylated whereas the higher molecular weight forms show almost no glycosylation (6); The function of O-glycosylation in NSP5 is unknown but this type of modification has been demonstrated to have a role in the regulation of the phosphorylation of cellular proteins (105) and this could be also the role in NSP5.

All NSP5 bands that appear on a SDS- PAGE are phosphorylated. Indeed, treatment of hyperphosphorylated NSP5 with phosphatases causes the disappearance of the higher molecular weight forms with a complete reduction of the migration of NSP5 to the only 26kDa band (figure 3 lane 4). However the 26kDa band is still phosphorylated showing that some of the phosphorylation sites are resistant to phosphatases (6, 195). Partial acidic hydrolysis of NSP5 obtained from infected cells followed by thin layer electrophoresis of the phospho-aminoacids demonstrated that NSP5 is phosphorylated only on serine and threonine (6), even though another study has suggested that only serine residues are involved in the phosphorylation events (22).

Many studies have tried to investigate how the phosphorylation of NSP5 takes place and it has soon become evident that this is a complex and multi step event.

If a construct expressing NSP5 is transfected in cells in absence of other viral proteins, this produces only the bands of 26 and 28kDa of NSP5 and, in very low amount, the higher mw forms (6). Similar results have been obtained through the use of a baculovirus system where again the hyperphosphorylation of NSP5 was very limited. Similarly, *in vitro* kinase assay of a bacteria-produced NSP5, caused the phosphorylation only of the 26kDa form.

Interestingly, okadaic acid, an inhibitor of PP1 and PP2A, caused the appearance of higher molecular weight forms of transfected NSP5 in absence of other viral proteins (23). This suggested the hyperphosphorylation of NSP5 to be independent from the presence of other viral proteins and that a cellular cofactor was necessary for NSP5 hyperphosphorylation. In agreement with this kind of interpretation was the result that Staurosporine, an inhibitor of protein kinase C (PKC) known to block also a wide variety of protein kinases, was partially able of blocking the hyperphosphorylation of NSP5 in virus infected cells (23).

Further studies have subsequently demonstrated the process of hyperphosphorylation of NSP5 to be more complex. When NSP5 is immunoprecipitated from extracts of [^{35}S] methionine labelled-, DSP crosslinked- and virus infected- cells, it appears to be interacting both with NSP2 and VP1. If these extracts are subjected to in vitro phosphorylation a substantial increase in the formation of the hyperphosphorylated bands is obtained if compared with non cross-linked cells (5). This has suggested that, in contrast with previous results, the presence of other viral proteins has a role in the hyperphosphorylation of NSP5. In agreement with this kind of experiment, when NSP5 and NSP2 are expressed in absence of other viral proteins in eukaryotic cells, a clear increased phosphorylation (28 kDa) and hyperphosphorylation (32-34 kDa) of NSP5 is obtained (Figure 4). This demonstrated that NSP2 activates NSP5 hyperphosphorylation in vivo most likely as a consequence of their interaction (5).

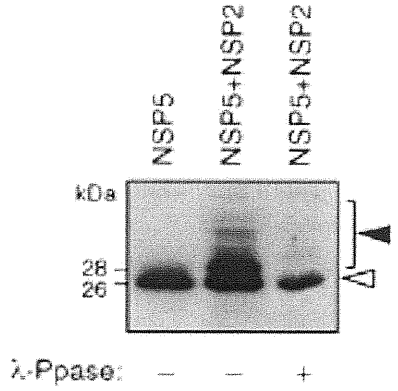


Figure 4: Anti-NSP5 western immunoblot of cellular extracts of MA104 cells transfected with pT7v-NSP5 or co-transfected with pT7v-NSP5 and pT7v-NSP2, as indicated. λ-Ppase treatment of the extract was performed before PAGE. Open and solid arrowheads indicate the NSP5 26 kDa precursor and phosphorylated forms respectively (5).

In order to better understand the process of hyperphosphorylation, NSP5 deletion mutants have been used. NSP5 have been arbitrarily divided into five regions (from 1 to 4 and a tail T). (Figure 5).

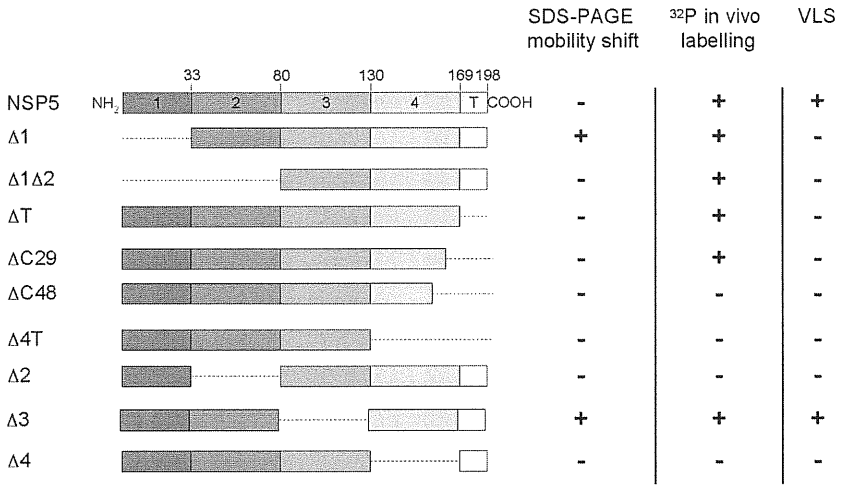


Figure 5: Schematic representation of the NSP5 mutants constructed. The relative amount of Ser/Thr for region 1,2 and 3 is 52, 23 and 30%, respectively. The ability of each protein to produce mobility shift, to be phosphorylated *in vivo* and to form VLS is also indicated. Modified from (80).

In place of NSP2, some deletion mutants of NSP5 showed to behave as good substrates or activators of the phosphorylation process. Deletion mutants lacking either region 1 or 3 or both (Δ1, Δ3 or Δ1/3) efficiently induce PAGE mobility shift in an *in vitro* translated deletion mutant such as Δ1 or Δ2. Similarly, *in vivo* coexpression experiments, have shown that the same deletion mutants Δ3 or Δ1/Δ3 were able of causing the phosphorylation of the mutant Δ2 (Figure 6).

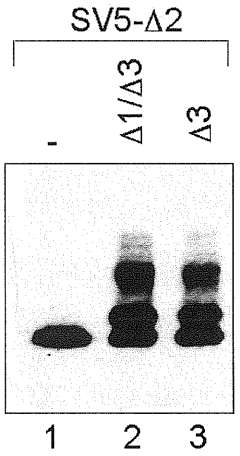


Figure 6: Coexpression of SV5-Δ2 with or without the activators Δ1/Δ3 or Δ3 as indicated. Δ2 was visualised with an anti- SV5 sera (71).

This has suggested that regions 1 and 3 have an inhibitory effect on the phosphorylation of NSP5. Interestingly, the same amount of histidine tagged deletion mutant protein purified from transfected cells extract on a nickel column, did not show the same phosphorylation activity of the cellular extract containing it, thus showing that NSP5 is not itself a kinase but has the capability of promoting the activation of cellular kinase(s) (73).

Phosphorylation and capability to activate a cellular kinase appear to be distinct characteristics of NSP5. This has allowed to uncouple the substrate and activation functions of NSP5 in experiments of coexpression of various NSP5 mutants. The activation process has been localised to region 2 and in particular to serine 67. This serine needs to be phosphorylated for NSP5 to become an activator. Once phosphorylation of Ser-67 has taken place, both regions 1 and 3 do not hinder further phosphorylation on NSP5 any longer. Probably these domains inhibit Ser-67 phosphorylation blocking its accessibility. This interpretation is supported by the fact that an NSP5 mutant where Ser-67 is substituted by an aspartic acid (S67D), that mimics a phosphorylated serine, is able of getting phosphorylated when expressed alone in cells, whereas NSP5 wild-type or mutants where the same serine is substituted by an alanine (S67A) do not show the same phenotype (Figure 7).

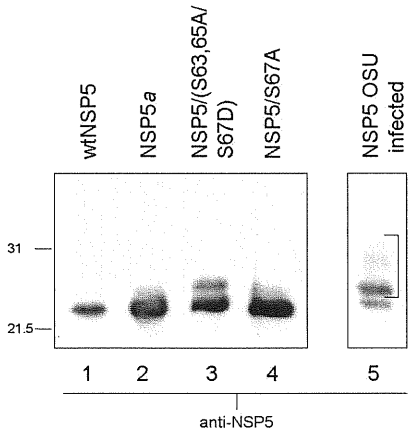


Figure 7: Expression of full length NSP5 and NSP5 point mutants. Lane 5 correspond to NSP5 from rotavirus-infected MA104 cells. Samples were migrated in a 15% SDS/PAGE and visualised by Western blot (71).

In addition this work has shown that the region where Ser-67 is localised is similar to the consensus phosphorylation site for protein kinase 1 alpha also called CK1α. Indeed, during *in vitro* phosphorylation experiments, a recombinant CK1α from zebrafish caused the phosphorylation of a NSP5 peptide of 14 residues encompassing the recognition motif for CK1α, whereas a peptide in which the position corresponding to Ser-67 was mutated to

alanine did not undergo phosphorylation. In addition similar results were obtained when bacteria-expressed NSP5 mutants were used (71).

CK1 represent a separated group within the superfamily of serine/threonine specific protein kinases. They are ubiquitously expressed in eukaryotic organisms and evolutionarily conserved with several elements found also in yeast. The members of the family act as monomers, do not use a cofactor, are constitutively active and use ATP as sole font of phosphate. Up to now seven members of the family have been identified (α, β, γ1, γ2, γ3, δ and ε) that span from a molecular weight of 37kDa (CK1α) to 51kDa (CK1γ3), furthermore some of these members generate different proteins through alternative splicing (33). All CK1 family members contain a highly conserved kinase domain but differ significantly in their N-terminal and C-terminal non catalytic domains and have been suggested to have different functions. However, *in vitro* studies with model peptides have not shown a significant target preference for the various isoforms, suggesting that substrate preferences are due to other characteristics such as cellular localisation and differential expression (203). In general these proteins were shown to be involved in a large range of cellular processes such as circadian rhythm, membrane transport, cell division, apoptosis and Wnt pathway (126). In addition, CK1 was found to be involved in the phosphorylation of viral proteins of various types of viruses such as the simian virus 40, a virus with a dsDNA genome, the respiratory syncytial virus, a non segmented negative strand ssRNA virus, and the influenza C virus that contains a segmented ssRNA genome (35, 36, 141).

The 18aa of the C-terminal domain of NSP5 are necessary for both the activator and substrate activity (71). This is in agreement with the fact that the tail is responsible for the NSP5 dimerisation capability (73, 235). Altogether these data allowed to design a model of the phosphorylation of NSP5 (Figure 8). In this model NSP5 interacts with NSP2 through the N-terminal region, in agreement with the finding that deletion mutants of NSP5 lacking the N-terminal region do not interact with NSP2 (80). This interaction favours the phosphorylation on Ser67 by CK1α and this occurs concomitantly or followed by dimerisation of NSP5 through the C-terminal region. The NSP5 phosphorylated on ser 67 activates the other NSP5 molecule of the dimer making it susceptible to hyperphosphorylation by the same CK1α or another cellular kinase (71).

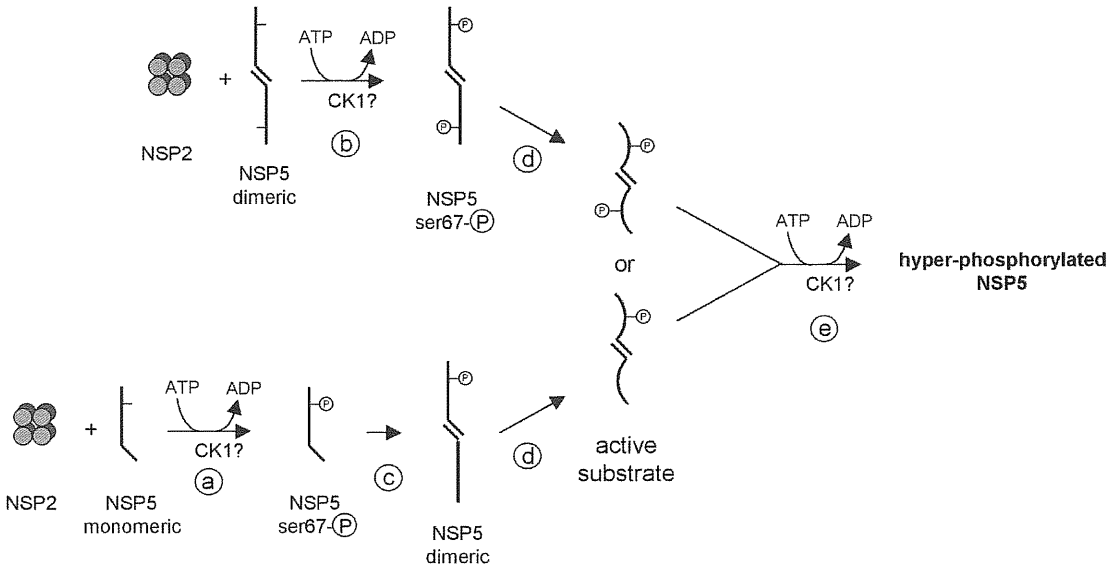


Figure 8: Model for rotavirus NSP5 hyper-phosphorylation. Interaction of NSP2 with monomeric (a) or dimeric (b) NSP5 promotes phosphorylation of Ser67 by CK1 (in either one or both subunits), which in the case of the monomer may induce dimerisation (c). A phosphorylated Ser67 in the dimer induces conformational changes (d) to render the other partner molecule (and probably both) susceptible to hyper-phosphorylation by the CK1-like cellular kinase (e) (71).

Apart from the involvement of NSP2 in the phosphorylation of NSP5, another interesting characteristic of these two proteins is the fact that they co-localise in infected cells in viroplasms. Analysis of viroplasm formation in infected cells has shown that NSP5 is localised in the outer part while NSP2 has a more internal localisation (72). In addition, when co-expressed, they are able to give rise to spherical structures that resemble very much the viroplasms and that for this reason have been called Viroplasm Like Structures (VLS) (Figure 9) (80).

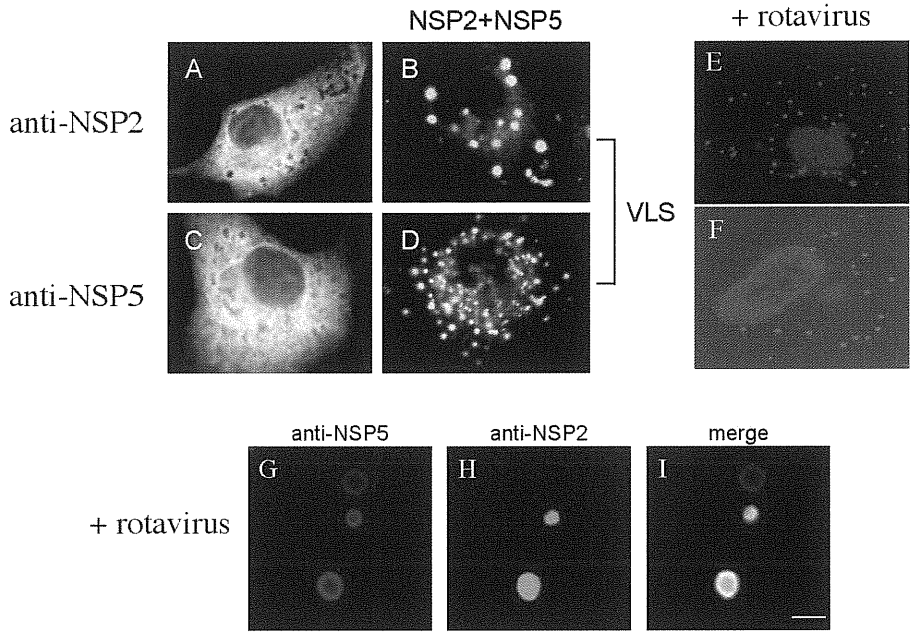


Figure 9: Immunofluorescence of viroplasms or VLS using specific sera against NSP5 or NSP2. (A-D) Formation of VLS: the two proteins were analysed by immunofluorescence microscopy using specific antibodies for NSP2 or NSP5 as indicated, in cells transfected with either NSP2 (A) or NSP5 (C) or co-transfected with NSP2 and NSP5 (B,D) (80). (E-F) Viroplasms in infected cells: cells were SA11 infected for 5hrs and analysed by immunofluorescence using specific antibodies as indicated. Nuclei stained with Hoechst dye (blue). (G-I) Amplified images of viroplasms in rotavirus infected cells: double immunofluorescence using an anti-NSP5 (red) and an anti-NSP2 (green) sera. Images obtained with confocal microscopy, it can be observed the outermost localisation of NSP5 respect to NSP2 (72).

An analysis of the deletion mutants of NSP5 has shown that those that are able of causing the formation of VLS contain the N and C terminal regions of the protein and are those that get hyperphosphorylated (80). This suggests that also for viroplasm localisation is necessary the phosphorylation of the protein. However the necessity of interaction between NSP2 and NSP5 to form VLS has been questioned by a work that has demonstrated that if the N-terminus of NSP5 is 'blocked' by fusion with a tag such as GFP or HA, NSP5 is able by itself of causing the formation of structures similar to VLS in absence of NSP2. In this study it was shown that only the C terminal 68 residues of NSP5 fused to GFP at the N terminus are necessary for the formation of these structures whereas the fusion at the C terminus causes the lack of formation of this kind of VLS (168).

Apart from interacting with NSP2, NSP5 interacts both with the viral polymerase VP1 (5) and with VP2 (18). Interestingly, when VP2 is co-expressed together with NSP5 in insect cells, this causes the formation of large inclusions that contain both proteins. In addition NSP5 does not interact with DLPs, but strangely it interacts with cores and VLP formed by VP2 and VP6 or by VP1, VP2, VP3 and VP6, maybe because in these structures the limited

number of VP6 trimers allows the concomitant interaction of VP2 and NSP5 or because VP2 has a stronger interaction with VP6 in DLPs than in VLPs. In agreement with the alternative role of the two proteins, NSP5 is able to dislodge VP6 from VLP2/6 and VP6 seems to hinder in part the interaction between NSP5 and VP2 suggesting a competition between these two proteins for the binding to VP2 (18).

Since NSP5 localises in viroplasms, where viral replication and transcription take place, the capability of NSP5 of binding ss and dsRNA have been investigated. These studies have shown NSP5 to have unspecific affinity for both ss and dsRNA suggesting the protein could have a role in recognising secondary and tertiary structures of the mRNAs that have to be packaged into the forming core and together with NSP2, that has NTPase and ssRNA binding activity, unwind these structures (243). It is interesting to underscore that the only other viral protein that interacts with dsRNA is VP2, even if also this protein has a higher affinity for ssRNA (129).

Despite all the information that have been obtained up to now about the protein, no clear function for NSP5 has been discovered so far. In addition no homology between NSP5 and any other protein has been found so far, making more difficult the understanding of its function.

1.4.6. NSP6

NSP6 is the smallest protein produced by rotavirus genome. It is encoded by an alternative ORF of segment 11 characterised by a stronger start codon motif than the one of NSP5. This ORF gives rise to a protein of 92 residues and 11kDa in SA11 infected cells (159). NSP6 has been shown to be expressed at very low level in infected cells and to localise in viroplasm (159). Through the use of two hybrid system it has been demonstrated that NSP6 interacts with the C terminal tail of NSP5 (93, 159, 235), even if interaction *in vivo* of the two protein in co-immunoprecipitation experiments from infected cell has not been obtained (235). The possible interaction of NSP6 with viroplasms and its low amount raise the possibility of a regulatory role of NSP6 in viral replication or, since it interacts with the region of NSP5 involved in dimerisation, it could have a role in this process (235). However the rotavirus group A strain OSU contains a truncated form of NSP6 that reduces the ORF to only 51 amino acids. In addition other two group A strains (Alabama and Mc323) and all the group C

strains do not encode for NSP6 (235) suggesting that the role of this protein is not essential for the replicative cycle of the virus.

1.5. STRUCTURE OF VIRAL GENOMIC SEGMENTS

The genome of rotaviruses consists of 11 discrete segments of dsRNA that have a size range of 0.6 to 3.3 kbp with a total average size of the genome of approximately 18550 bp (63). Genomic segments can be easily separated by polyacrylamide gel electrophoresis based on their length and are numbered following the order of migration being the slower (and longer) segment numbered as 1. Based on their size the genomic segments of group A rotaviruses fall into 4 classes with a 4,2,3,2 distribution: 4 high molecular weight segments (1-4), two middle size segments (5-6), a triplet of segments of very similar molecular weight (7-9) and two small segments (10-11) (Figure 1A).

Genomic segments are characterised by a series of common features:

- the genome is rich in A+U (58%-67%)
- the dsRNAs are base paired end to end
- the positive strand is capped (as described in § 1.6.2.)
- all genomic segments contain untranslated regions (UTRs) of variable length at the 5' and 3' that are important for the processes of transcription, replication and packaging
- each segment contains an ORF which is usually characterised by a strong initiation codon based on the Kozak's rules, although some of the genes contain additional in-phase ORFs (genomic segments 7, 9 and 10) or out-phase alternative ORFs (genomic segment 11).

One of the most interesting features of rotavirus is the fact that, like all the viruses that have a segmented genome, they must have a mechanism of sorting and packaging of the genome. Even though this mechanism is far from being understood, it is evident that the 11 positive strand RNAs that are used as templates for the synthesis of the double strand genomic segments and packaged must contain cis-acting signals that allow their recognition by the polymerase VP1 and their sorting. Whereas signals for replication and translation have been identified (as described in § 1.6.3, and § 1.6.2.), those necessary for packaging and sorting must still be elucidated, but it has been suggested that secondary structures, rather than primary ones, could be involved. Predicted secondary structures of some viral positive strand

RNAs indeed have been shown to contain 5' 3' complementary sequences that form a panhandle structure and one or more conserved stem-loop sequences at the 5' 3' termini that could be important for the processes of packaging. In particular the stem-loops differ in each positive strand viral RNA and could have a role as signals for packaging (183).

1.6. THE VIRAL REPLICATIVE CYCLE

1.6.1. Cell binding and entry

The first interaction between the rotaviral virion and the target cell membrane is clearly a pivotal step in the rotavirus replicative cycle and it is a very extensively studied process because the understanding of this process is tightly related to the comprehension of characteristics of the virus cycle such as its tropism for specific cell types and the process through which the virus is intracellularly activated.

In the last few years a series of works have shown that many membrane proteins are able of interacting with the outer shell proteins of rotavirus (VP4 and VP7) and have brought to the idea that virus entrance is a multistep process where several sequential interactions are necessary for rotavirus binding and internalisation.

Rotavirus strains can be divided, with regard to their cell attachment capability, into neuroaminidase-sensitive or -resistant strains. The first are those strains whose first contact with the target cell occurs through the interaction of the rotaviral protein VP8* with sialic acid residues on the cellular membrane (81), whereas the resistant strains do not bind sialic acid and their interaction with the target cell is caused by the recognition of other membrane components. From a medical point of view these second kind of rotaviruses are more interesting since most, if not all, human rotaviruses are neuroaminidase resistant (46).

Apart from the difference in the first cellular contact, it is thought that the subsequent steps in virus attachment are identical in the neuroaminidase-sensitive and resistant rotaviruses.

VP5*, as previously mentioned, is able of interacting with the integrin $\alpha 2\beta 1$ through the sequence DGE; this is considered to be the first receptor for neuroaminidase resistant rotaviruses and the second in the case of the sensitive ones suggesting that $\alpha 2\beta 1$ can function either as first binder or as second interactor (51, 106). After this first contact rotavirus interacts with at least other three cellular receptors: the integrin $\alpha v\beta 3$ binds VP7 and it has been shown that VP7 contains a non conventional sequence (CNP) that recognises it; the integrin $\alpha x\beta 2$, that is as well recognised by VP7 through the sequence GPRP (100), and the Hsc70 that interacts with VP5* (102, 255).

It has been suggested that the interaction between rotavirus and the cell membrane occurs inside lipid rafts (103). In agreement with this view all the above mentioned receptors are associated with lipid rafts. Furthermore the depletion of cholesterol from the cell membrane as well as the inhibition of N-glycosylation and of glycolipids synthesis, all processes that disrupt rafts, can severely impair virus infectivity (103).

After binding, the second step of virus entry is internalisation. It has been shown that virus entry does not occur at 0° or 4°C indicating that the process is active (149). Furthermore it is rapid, indeed proteolitically activated rotaviruses enter with a $t_{1/2}$ of 5-10 min. The internalisation signal event is not known, but it has been suggested it could be mediated by integrins, since their β1 domain is known to be involved in cell signalling between the extracellular and intracellular compartment and could induce a signal for virus internalisation (106). Also Hsp70 has been suggested to have a role in the internalisation process, since members of this family are known to be involved in translocation across membranes and in assembly and disassembly of oligomeric complexes (255).

Two types of virus entrance have been proposed: direct cell membrane penetration and endocytosis. Ultrastructural images of rotavirus particles inside endosomes, as well as images suggestive of direct cell membrane penetration, have been obtained (149, 226) and up to now there is no clear evidence about the actual virus entry mechanism. Recently, through the use of pharmacological and biochemical approaches, it has been shown that virus entry occurs through a non-clathrin non-caveolae cholesterol sensitive dynamin dependent vesicular pathway (212). Dynamin is a protein shown to be necessary for the release of vesicles from the plasma membrane and it has been shown to be involved in cell entry of several viruses. But up to now no clear evidence about the actual internalisation pathway of the virus has been obtained and it is possible that more than one pathway is used.

1.6.2. Virus uncoating and transcription

Upon virus entry the first event known to occur is the uncoating of the virus with the loss of the outer layer. This is an important step since it triggers the transcriptional activity of the virus with the production of all viral mRNAs.

It has been suggested that the decrease of several orders of magnitude in the Ca^{2+} concentration between the extracellular environment and the intracellular one is responsible

for the uncoating of the outer layer of the virus and activation of the viral transcriptase (49, 149), even if this hypothesis is still controversial (54). The capability of both VP7 and VP5* of solubilising membranes could have a direct role in the process of uncoating (37, 61, 69, 91). In a possible model the critical Ca^{2+} concentration inside early endosomes is decreased by diffusion caused by the great concentration difference with the cytoplasm (1mM in the endosomes and 100nM in the cytoplasm) (207, 209). In addition another study has shown that the rapid loss of Ca^{2+} from the endosomes could be driven by the electrical gradient generated by the H^{+} -ATPase pump present in the endosomes (39). The decrease in the Ca^{2+} concentration would allow the release of the viral outer layer proteins that in turn would solubilise the endosomal membrane thus causing the release of the DLP. However there is no agreement on this view, for example it has been suggested that VP5* cannot be responsible of the solubilisation step, because it can only transiently permeabilise membranes and its role would consist in producing small pores in the membrane that would allow the efflux of the Ca^{2+} ions (69).

The loss of the outer shell activates the transcriptional activity of the virus with the production of mRNAs shortly after viral infection; the process causes an increase in time of mRNA production until 9 to 12 hours from virus infection, after which the synthesis begins to decrease (224). A delay of three hours from viral infection before a detectable quantity of mRNA is produced has been observed and attributed to the necessity of accumulation of the viral proteins necessary for the assembly of the transcriptase particles for the secondary transcription by newly formed DLPs (224).

The transcription is conservative leading to the formation of mRNAs that are identical to the plus strand of the 11 viral dsRNAs. It is a very complex process since during each cycle of transcription the dsRNA must move around the polymerase, unwind, separate, rejoin and rewind for the next cycle of transcription. Concomitantly the nascent mRNA must be capped and extruded through the channels at the 5-fold axis of the virion.

The synthesis of viral transcripts is mediated by the concerted action of the endogenous viral polymerase VP1, that behaves as a transcriptase, and of VP3. The inner virus shell, composed by VP2, is necessary for the transcriptional machinery to be active as well. The N terminus of VP2 has been shown to be able of binding ssRNA, VP1 and VP3. Furthermore VP2 is in close association with the genomic dsRNA and is considered to be the necessary scaffold protein for the assembly of the internal viral core (129, 257). Since the RNA template during transcription is double stranded, the necessity of a helicase activity to unwind the

dsRNA has been suggested. Unfortunately up to now no helicase activity has been identified in rotavirus but has been suggested to be attributable either to VP2 or to VP3 (133, 192). Also VP6 has been shown to be necessary for the replicase activity of the virus. I*n vitro,* VP6 mutants, that were not able to assemble with the viral core, could not restore the transcriptase activity. The hydrophobic interaction between VP2 and VP6 is crucial for the transcriptase activity as shown by DLPs, in which the VP6 trimer has additional negative charges in the region of interaction with VP2 that loose their transcriptional capability (38). Since VP6 does not interact with the viral polymerase VP1, it has been suggested that the interaction between VP6 and VP2 causes a conformational change of the core structure necessary for the transcription apparatus to work properly (38). Consistent with this view only DLPs are infective when liposome transfected into cells (16). On the contrary the transcriptase activity is latent in the TLPs and can be activated *in vitro* by treatments that remove the outer capsid proteins and release DLPs such as chelating agents (49) or heat shock (223). Interestingly TLPs are not strictly transcriptionally incompetent, they are capable of transcribing short 5-7nt long capped mRNAs showing that polymerase and capping enzyme are functional, but they are unable of continuing the elongation process of transcription and producing full length mRNAs (135). This behaviour resembles the one of transcriptionally incompetent DLPs when complexed with Fab fragments derived from monoclonal antibodies against VP6, suggesting that the presence of the outer capsid probably causes a conformational change in the viral architecture that prevents transcripts to be elongated and cause in this way transcription to occur only in the appropriate location (134, 135, 234).

The mRNAs produced are capped by the addition of $^{m7}GpppG^{m}$ at the 5', but do not contain a poli-A tail. The process of capping occurs in four steps:

1) phospho-hydrolysis to remove the terminal orthophosphate at the 5' end of nascent mRNA (pppG → ppG + p_i)

2) attachment of a guanine nucleotide to the 5' end (ppG + GTP→ GpppG + pp_i)

3) attachment of a methyl group coming from S-adenosyl-L-methionine (SAM) to the N^7 position of the G cap (GpppG + SAM → $^{m7}GpppG$)

4) second attachment of a methyl group (m^7GpppG + SAM→ $^{m7}GpppG^{m}$)

As a result the process necessitates four different enzymatic activities. It has been shown that VP3 is the enzyme responsible for most of these reactions, being the viral methyl-/guanyl-transferase, so far it has not been possible to attribute the phosphohydrolase activity to any viral protein (42, 145, 180, 191).

It has been demonstrated that viral mRNAs are released through the 12 type-I channels at the icosahedral five-old axes of the DLPs (137). It is worth noting that the complexes VP1/3 localise immediately below this channels in the core of the virion (201).

Through cryomicroscopy it has been possible to visualise the process of active transcription of DLPs in vitro. This kind of study has showed that several mRNAs can be released simultaneously from the DLPs (137) confirming the fact that DLPs are able of transcribing concomitantly many different single stranded RNA molecules (223). Another interesting information obtained by cryomicroscopy imaging has been the fact that viral particles remain intact during transcription confirming the process to be conservative (137). This is also in agreement with the fact that viral particles are able of continuous transcription. In any case the mechanism through which the genomic segments are organised in order to allow reinitiation of virus transcription has not yet been elucidated.

1.6.3. Translation

Studies of the kinetics of viral polypeptide synthesis showed that proteins are produced 2-3 hours after infection with a concomitant inhibition of cellular polypeptide synthesis (76, 161) and that the process continues until around 10 hours when it drops to low levels (23).

As previously mentioned viral mRNAs are characterised by a $^{m7}GpppG^{m}$ cap at the 5' like the one of eukaryotic messengers, but do not contain a poli-A tail. Whereas the capped 5' region can be recognised by the cap binding protein of the translational elongation complex eIF4E, the lack of a poly(A) tail renders the viral mRNAs not suitable for translation and exposed to degradation by cellular exonucleases. Indeed, these mRNAs are not efficiently translated in absence of viral infection unless NSP3 is present even at low levels (242). The function of NSP3 has been suggested to be the substitution of poly(A) binding protein (PABP) in order to localise the 3' end of viral mRNAs in the right position of the initiation complex of translation. This would guarantee an efficient translation of viral mRNA (190). Interestingly the strategy used by NSP3 to bind the 3' of mRNAs is completely different from the one used by PABP. The last one interacts with different recognition sequences on the mRNAs with poly (A) tail, whereas NSP3 only interacts with the recognition signal at the end of mRNA with high affinity and completely wrapping it in a tunnel like structure (62). This type of interaction probably is necessary in order to avoid the degradation of viral mRNAs that do not

contain a poly(A) tail and to allow an efficient and selective translation of only viral mRNAs for the duration of the infection.

1.6.4. Virus replication and RNA packaging

Replication and packaging of the viral genome is one of the least understood processes in the rotavirus replication cycle and one of the most studied since its comprehension and dissection would allow the construction of recombinant rotaviruses with designated mutations opening new possibilities for the study of rotavirus replicative cycle and for the construction of vaccines.

Upon viral entry and outer layer loss, ssRNAs of positive sense are made in the DLPs that can function both as mRNAs for the production of viral proteins, as mentioned above, or as template for the production of dsRNA by synthesis of the complementary minus-sense strand. This last is a very complex process since the rotavirus genome is composed of 11 segments that must be all replicated, sorted and packaged in order to have the formation of a complete virion with all the 11 segments. Unfortunately the signals necessary for the right assortment of the viral segments are far from being understood.

The replicative process begins around three hours after viral infection and increases in time reaching maximal levels around 9-12 hours (224).

Studies on viral particles isolated from infected cells with replication capability, called replication intermediates (RIs), have shown that these particles contain the structural proteins that are also found in single shelled particles and DLPs (VP1, VP2, VP3 and VP6) and, in addition, the non structural proteins NSP2 and NSP5. Since all these proteins are known to localise in viroplasms, this has led to the suggestion that these structures are the site of replication of the virus and that the processes of replication, packaging and assembly of the virus occur concomitantly (87, 182, 188). This is also in agreement with the fact that no free dsRNA has been found in infected cells. *In vitro* studies have shown that the non structural proteins are actually not necessary for viral replication and that the minimal proteins necessary in order to obtain replication in vitro of viral mRNAs are VP1, the viral polymerase, and VP2, the scaffold protein that constitutes the inner layer of the virus (44, 258). However if the two proteins are sufficient for viral replication, they are not for RNA packaging, suggesting that the requirements for packaging are more complex than those for replication. It

might be that interactions among the secondary structure of viral plus strand RNAs give the necessary signal for packaging, alternatively other proteins localised in viroplasms could have a role. It has been suggested that NSP2 with its helix destabilising ability and its capability of binding to ssRNA, alone or in association with NSP5, could be involved in the packaging process (114, 231). Additional evidence that NSP2 could have a role in packaging comes from temperature sensitive studies, where a NSP2 mutant, produced mostly empty particles at the non-permissive temperature (41, 205).

Based on the existing information three models of virus replication and packaging have been suggested (183):

1. *precore precursor model:* each positive strand RNA corresponding to one of the genomic segment interacts with the polymerase VP1 and the capping enzyme VP3 prior to core assembly; the interaction among these 11 complexes could act as nucleation site for the assembly of the inner layer by VP2

2. *RNA pentamer precursor model:* this model differs from the previous for the fact that each complex of VP1, VP3 and the RNA segment assemble with 10 molecules of VP2 to form a functional unit of the virion, and afterwards these pentameric complexes assemble among them to form the core structure

3. *Empty core precursor model:* in this model the core shell is assembled first and afterwards the viral RNA are packaged

An important tool for the identification of the RNA sequences necessary for replication has been the use of open cores (44). This system is based on the disruption of the core particles through their exposure to hypotonic buffer. These 'open cores' release the genomic segments, but they are still able, in presence of Mg^{2+} and of the four ribonucleotides, of catalysing the synthesis of dsRNA using as template cDNA-derived rotavirus mRNAs. Through the use of this method and of mRNAs that contained mutations it has been possible to locate the regions and structures of mRNA necessary for viral replication.

Three regions have been found to be important:

a. The first signal is represented by the 3' consensus sequence (3'CS) that in group A rotaviruses is: 5'-UGUGACC-3'. This is the most important and critical *cis*-acting signal for replication, indeed the sequence is very conserved. The first experiments with open cores had shown that the last 26 nucleotides of viral positive strand RNA were important for replication (44). Subsequent studies have restricted the required sequence to the only last seven nucleotides (184, 246). It has been shown that the last two nucleotides (CC-3') are of primary

importance for initiation of replication while the other nucleotides are less important (40). In addition it is necessary that the 3'CS is single stranded in order to function efficiently as replication signal (43).

b. A second region lies upstream the 3'CS at the 3' end of viral mRNAs; this sequence seems to have an enhancing role on replication (184). It has been shown for genome segment 8 that several independent signals are localised in this region and are recognised by the viral polymerase (236).

c. A third region that localises on the 5' terminus has been shown to enhance replication. This region does not contain conserved sequences and its role in replication is not clear. However it has been proposed that the secondary structure formed by this region is important for replication: this 5' region would base pair with a region at the 3' end of the mRNA to form a panhandle structure from which the 3'CS would extend as single strand (43, 184, 246).

In addition, the predicted secondary structure of viral mRNAs contain one or more stem-loop structures located at the 3' and 5' of the mRNA that are different for the various mRNAs and have been proposed to have a role in packaging and assortment (183).

It is of interest to note that the last four nucleotides of viral mRNAs are also the recognition sequence for NSP3 and necessary for the translation of the viral mRNAs (45, 194). Actually a recent study on genome segment 5, coding for the non structural protein NSP1, has shown that the 3'CS (3'-UGUGACC-5') is not the optimal one for replication: addition of an 'a' in the 3' sequence (5'-UGUGAaCC-3') increases the efficiency of replication of the segment, but decreases the efficiency of translation showing that the 3'CS is probably a sub optimal sequence for replication retained because necessary for optimal translation (123).

As described previously, the interaction between NSP3 and the 3' end of viral mRNAs occurs in a basic tunnel of NSP3 closed at one end (62). This has lead to the question how these mRNAs could be used by the replicase machinery since it needs to recognise the same 3' end that is so tightly hidden inside NSP3. This question has been answered through the use of RNA interference: it has been shown that the block of production of a viral protein that is not involved in RNA replication (i.e. VP7) through the degradation of the corresponding mRNA did not affect the replication of the very same segment. This has suggested that the viral mRNAs involved in protein production were not the same ones used for viral replication and that these ones were protected from the action of RNA interference, probably because of their localisation into viroplasms (221).

1.6.5. Virus morphogenesis and release

The morphogenesis of rotavirus is a rather unusual process that differentiates from the one of other viruses from the fact that it does not follow a classical exocytic route to be released at the apical membrane of intestinal cells. In the last few years a series of studies have begun to gain new information on the process even though a clear understanding has not yet been obtained.

The first steps of rotavirus morphogenesis occur inside viroplasms where the core particles that contain the viral structural proteins VP1 and VP3 and the 11 genomic segments surrounded by the inner layer protein VP2 are assembled. After assembly of the core particle this acquires the intermediate layer composed by protein VP6. VP6 is known to localise in viroplasm in a distribution quite similar to the one of NSP2 and NSP5 (92) even though other reports have shown VP6 to localise in the cytoplasm between the viroplasms and the ER (188). The assembly of the intermediate layer is thought to occur concomitantly with the exit of particles from viroplasms (78). In a recent study RNA interference against NSP4 has shown to have an effect also on the formation of the intermediate layer. Indeed, in absence of NSP4 little or no viral particles (DLPs and TLPs) were formed, suggesting a more direct involvement of NSP4 in the assembly of the VP6 layer (147). Furthermore the distribution of VP6 was altered since it was not anymore found to localise in a viroplasmic or peri-viroplasmic region, but to form filamentous structures that extended to the periphery of the cell (147).

Upon formation DLPs are known to bud into the ER through the interaction with the 20 extreme residues of the cytoplasmic tail of NSP4 with VP6 (11, 172, 233). It is interesting to note that even though NSP4 is known to heterotrimerise with VP7 and VP4 (152), the two structural proteins do not have a role in the viral budding in the ER, indeed the process is not blocked by the depletion of either protein with the use of RNA interference (147).

The last steps of viral morphogenesis occur in the ER. Upon entry into the ER the maturing virion acquires a transient envelope where VP7 localises in the interior possibly relocating across the membrane (196) and the outer layer of the virus is formed. During the process of maturation the transient envelope is lost by a process not well understood. The use of tunicamycin, an inhibitor of the ER N-linked glycosylation process, results in the block of

viral particles as membrane-enveloped (59, 187, 210). Both viral proteins that localise in the ER, VP7 and NSP4, are glycosylated but since in nature exist rotavirus strains that are able to produce fully infectious particles despite the fact that VP7 is not glycosylated, it has been suggested that the inhibition of membrane removal is due to the lack of NSP4 glycosylation (59, 187). However another report has shown that NSP4 glycosylation does not have an effect on virus maturation (163). In addition a more recent report has shown that also in the absence of VP7, viral particle assembly is blocked at the enveloped stage suggesting a role also for VP7 in this process (147). A central role in envelope removal seems to be exerted by the Ca^{2+} concentration inside the ER. Neo forming virions pass from the low concentration of calcium of the cytoplasm in the range of nM to the high concentration mM inside the ER and this change seems to have a central role in outer layer assembly. Indeed, a decrease in the concentration of Ca^{2+} in the ER results in block of viral particles in the enveloped state (162, 197). A decrease in Ca^{2+} concentration affects the processing of viral particles localised in the ER in a way similar to the effect induced by tunicamycin and it has been suggested that the decrease in Ca^{2+} levels and NSP4 glycosylation could be two connected processes: the lack of glycosylation could block Ca^{2+} mobilisation from ER by NSP4 or, conversely, a decrease in Ca^{2+} concentration could affect the glycosylation status of NSP4 (59, 162). Ca^{2+} decrease causes also a disruption of hetero-trimerisation among VP7, VP4 and NSP4 (197) and also this could affect envelope removal. In addition VP7 trimerisation is stabilised and dependent on Ca^{2+} (66) and it has been suggested that also the process of VP7 trimerisation and assembly in the presence of Ca^{2+} could be responsible of the envelope removal (147). All together all these information suggest that the transient envelope loss is a process that requires the presence of both NSP4 and VP7.

Interesting information about the virus release come from the use of Caco-2 cells. These are human intestinal epithelial cells established from a human colon adenocarcinoma that, after confluence, display many of the morphological and biochemical properties of mature enterocytes. These cells are polarised and contain two different plasma domains, the apical one characterised by a brush border and expression of intestinal hydrolases, and the basolateral one (189). Through the use of these cells it has been possible to demonstrate that in virus infected cells the neo formed virus is released almost exclusively on the apical pole in a non conventional vesicular transport process that bypasses the Golgi apparatus. In addition the process of virus release has been shown to occur in absence of cell lysis, an aspect that better fits the pathological findings of natural rotavirus infection (117) (described in § 1.7.).

In Caco-2 cells VP4 possesses an autonomous signal for association with rafts on the apical membrane of Caco-2 cells and, at later stages of viral infection, rafts are also enriched in infectious particles and NSP4 (55, 60, 215). A previous study had already shown the localisation of the spike protein VP4 at the plasma membrane very early after infection and its association with vesicular structures and with β-tubulin and microtubules (171). As previously mentioned, the fold of VP8* resembles that of galectin, a family of sugar binding proteins that reach the plasma membrane bypassing the Golgi and in association with rafts, suggesting a similar method of apical membrane localisation for VP4 (215). In addition drugs that interfere with rafts formation alter the migration of the virus and reduce its release (55). Based on these studies it was suggested that the last stages of virus assembly could take place in an extrareticular compartment in raft vesicles containing VP4 (55, 215). The fact that VP4 could assemble in later stages and not necessarily during the transit of maturing virions in the ER is also in agreement with results obtained through the use of RNA interference against this protein that have shown how, in absence of VP4, the outer shell is assembled and spikeless TLPs are formed (58). In this context it remains unclear what directs immature viral particles to rafts. Two obvious candidates are the only two ER resident rotavirus proteins VP7 and NSP4. In particular NSP4 has some characteristics that makes it a good candidate: it is able to heterotrimerise both with VP7 and VP4 (152), it is the only non structural protein that has been found to localise in rafts (55, 59, 215) and it interferes with the traffic of vesicles to the membrane in a pre-Golgi stage (250). This is a particularly interesting characteristic since rotavirus has been shown to be released in a vesicular system that bypasses the Golgi (117). Taken together all this information allowed Delmas and colleagues to propose a model for rotavirus release. In this model VP4 early after infection localises in raft vesicles that are transported to the apical membrane. The neo-formed enveloped DLPs move from the ER to contact sites between the ER and the Trans Golgi Network where they associate, probably through the activity of NSP4, with rafts vesicles where VP4 is localised. A fusion event between these two vesicles would cause in the last stage of virus infection the targeting of virions to the apical membrane (maybe through the galectin domain of VP4) and their release (60). Interestingly, as previously mentioned, VP4 interacts with Rab5 and its receptor PRA1 (75), two proteins involved in vesicular trafficking and fusion suggesting a possible role of these proteins in the fusion process. This is however an hypothetical model that does not take into account, for example, the data of localisation of VP4 in the region between the viroplasms and the ER (188) and its trimerisation with NSP4 and VP7 (152).

1.7. PATHOGENESIS OF ROTAVIRUS INFECTION

Rotavirus infection accounts for around 45% of severe diarrhoeal diseases in infants and young children both in developed and developing countries. Unfortunately the only vaccine so far licensed, Rotashield, was withdrawn from the market because of association with intussusception. The reason why Rotashield was causing this kind of bowel obstruction is unclear also because intussusception has not been associated with natural rotavirus infection. However this kind of event has emphasized the need for a better understanding of the mechanisms of rotavirus pathogenesis and interaction with the gastrointestinal tract.

Even though the disease is induced in young children of the age up to 2 years, individuals of all ages are supposed to be susceptible to rotavirus infection as demonstrated by the fact that immunocompromised adults can be chronically infected and suggesting that age restriction is not due to the disappearance of a specific receptor for rotavirus in the gastrointestinal tract in older individuals, but to the development of an immune response that protects from the infection. It has been shown that virus clearance is linked to the production of specific IgA and that individuals exposed to rotavirus infection develop acquired immunity (83). However there is no agreement on this view since other reports have suggested a difference in cellular signalling in young children that could account for the differences in infectivity (170).

Diarrhoea by rotavirus is considered to be mal-absorptive and caused by the transit of undigested osmotically active species in the colon where the incapability to absorb sufficient water leads to diarrhoea.

The small intestine is coated by cells that are generally divided in two types: enterocytes and crypt cells. Villus enterocytes are mature non dividing highly polarised cells, that are specialised in digestive and absorptive functions and, for this purpose, they synthesize a series of peptidases, proteases and other enzymes that are exposed on the luminal surface of the small intestine where they carry out their work. Crypt cell instead are the progenitor cells of enterocytes, lack the absorptive capability, but in turn secrete Cl^- ions so that the two types of cells have opposite functions where enterocytes have a more absorptive kind of function and the crypt cells a secretory one.

Rotavirus infects mature enterocytes in the middle and upper region of the small intestinal villi. It is not known if this tropism is due to a specific factor expressed on those cells or to the necessity of some intracellular mediator (169).

Rotavirus infection is associated to very mild lesions of the intestine and to practically no inflammation if compared with other intestinal pathogens. In many animal models a direct correlation between the disease symptoms and inflammation or lesions of the small intestine has not been found. For this reason rotavirus pathology has been correlated with other characteristics of viral infections different from inflammation and villi lesions. Viral factors associated with virulence have been searched through the use of reassortants. These studies have shown a correlation between virulence and some viral proteins, structural proteins VP3, VP4, VP6 and VP7 and the non structural proteins NSP1, NSP2 and NSP4 (169). In particular mutations of NSP4 have been associated with altered virus virulence supporting a role for NSP4 in virus pathogenesis (259). Furthermore it has been shown that NSP4 or the peptide $NSP4_{114\text{-}135}$, are able of inducing age dependent diarrhoea in mice with symptoms that strongly mimics the disease cause by rotavirus itself. It has been suggested that NSP4 act as an enterotoxin being the first described so far in viruses (14). NSP4 seems to act both intra and extra cellularly. The expression of NSP4 inside the cell upon viral infection, causes a disruption of Ca^{2+} homeostasis and the increase in Ca^{2+} permeability triggers a number of cellular processes such as disruption of the cytoskeleton with impairment of the microvillar structure, inhibition of the Na^{+} -solute cotransporter system and lack of expression of enzymes at the apical surface of enterocytes necessary for digestion. In total these events lead to a reduced absorptive capacity of the epithelium and, consequently, to malabsorption. Apart from its intracellular action in infected cells, it has been demonstrated that the cleavage product of NSP4 corresponding to residues 112-175 (so comprising the region with enterotoxic activity) is secreted from rotavirus infected cells soon after infection (260). This peptide has paracrine effect on uninfected cells, binds to an unknown receptor and triggers a phospholipase C-inositol 1,3, 5-triphosphate cascade that causes the release of Ca^{2+} from the ER.

Furthermore it has been shown that NSP4 causes an increase in the paracellular permeability to molecules of the polarised epithelium of villi, through the impairment of the functionality of tight junctions (227). According to a recent report, it has been found that secreted NSP4 interacts with extracellular matrix proteins even though a functional significance of these interactions is still unclear (27). Finally it has also been suggested that

the interaction of NSP4 with the enteric nervous system (ENS) could have a primary role in rotavirus pathogenesis. This system controls the gastrointestinal tract and functions rather independently from the central nervous system. It has been shown the ENS to have a primary role in the late stages of rotavirus pathogenesis. Drugs that block the action of ENS are able of strongly attenuate the effects of rotavirus diarrhoea. It has been calculated that in mice up to 67% of fluid loss is caused by the effect of activation of ENS (150).

In late stages of infection rotavirus causes cell death with the release of the virus in the intestine lumen and allows the infection of the neighbouring cells. In a few cycles this causes an extensive lysis of the enterocytes with a consequent shortening of the microvilli and a decreased capability of absorption. Furthermore concomitantly with this cell lysis an hyperplasia of the crypt cells can be observed which is accompanied by hyper-secretion that contributes to increase the diarrhoeal symptoms. However this kind of lesions appears late during the rotavirus infection and is responsible only for the last stages of the pathology.

It seems evident that rotavirus diarrhoea is the result of many factors that cooperate to the reduced capability of absorption of the surface of the intestine and to malabsorption.

1.8. ROTAVIRUS VACCINES

In August 1998 a rotavirus vaccine, called Rotashield, was licensed in the USA. Actually this was not the first attempt to obtain a vaccine against rotavirus. Twelve years before, another vaccine prepared by SmithKline-RIT had been tested in Finnish infants and showed to be able of protecting from infection. This vaccine, based on a bovine strain of rotavirus, laid the scientific foundation of the rotavirus vaccines that followed. Indeed it demonstrated that live oral vaccines were highly effective in protecting infants against rotavirus and that immunisation with naturally attenuated rotavirus strains did not cause symptoms of diarrhoea, but did provide cross protection against the disease. The development of this vaccine was discontinued, because it seemed to be less protective against rotavirus infection in developing countries and the data on protection in other settings were inconsistent. Similar results were obtained from other vaccines based on animal strains that were tested in the same period. This led to the pursuit of multivalent vaccines based on reassortant strains with outer capsid characteristics of the most common human strain serotypes (G1-G4) but the attenuated characteristics of animal strains. Rotashield from Wyeth-Lederle was the first of these

multivalent vaccines to be released on the market. The vaccine within three months from its release was recommended for the immunisation of all american infants by both the US Centres for Disease and Control and Prevention (CDC) and the American Academy of Paediatrics: in less than one year more than 600000 infants had received at least one immunisation.

In June 1999 the Vaccine Adverse Events Reporting System of CDC reported that 15 children developed intussusception (a potentially fatal bowel obstruction) after the administration of the vaccine. This caused the suspension from CDC of the recommendations for the vaccine while this association was better investigated. A series of studies confirmed the association even if a clear attributable risk has never been estimated. For this reason in October 1999, only a little more than one year after its release, Wyleth Lederle Vaccine and Paediatrics voluntarily withdrew Rotashield from the market. This decision of stopping the immunisation has been very controversial and remains a topic of debate. If preliminary data suggested a risk of intussusception of one case each 2500 children immunised, following studies decreased the risk to one in 9500 or even to one in 40000, when the vaccine was administered in the first two months of life, in agreement with the fact that the majority of cases of intussusception occurred in children aged 3 months or older when vaccinated. In spite of all this, in a meeting held by World Health Organisation (WHO) in 2000 to assess whether and how developing countries could introduce RotaShield in their vaccination programs, this possibility was refused; no government or institution wanted to take the risk of introducing a vaccine that had been withdrawn from the US market (248).

Nowadays two new vaccines are ready to be introduced into the market: Rotateq from Merk, a human-bovine reassortant, and GSK's Rotarix based on an attenuated human strain. Both vaccines are undergoing very large phase III trials (more of 60000 children/ trial) in order to be able of monitoring the possible occurrence of intussusception both in the developed and developing world, and showed up to now strong protection, even though there are no data about their performance in the poorest countries of Africa and Asia, where they are mostly needed. The fact that trials are performed both in developed and developing countries could help speeding up the time needed for the vaccine to be introduced in developing countries. Indeed when Wyeth-Lederle asked WHO for a global recommendation for RotaShield introduction also in developing world, this was refused because the efficacy of the vaccine in developing world had not been assessed. In any case it is of crucial importance to assess the behaviour of a live attenuated vaccine in a developing world environment since it

could be very different from the expected one in particular because of the high reassorting capability of rotavirus.

In parallel with the GSK and Merk's other vaccines are under clinical trial, interestingly some of them are made by manufacturers of developing country that have a strong and more direct interest in getting a efficacious protection against rotavirus and it is probable that one or more of them will arrive in the market in a couple of years. It still remains to be assessed whether a live oral vaccine based on an attenuated strain of rotavirus can protect against the enormous array of rotavirus serotypes and varieties and if it protects also in settings where children get infected with more unusual serotypes and mixed strains and are affected also by malnutrition and other diseases.

1.9. RNA INTERFERENCE

Higher eukaryotes have developed a series of innate antimicrobial defence mechanisms that are based on the capability of recognition of conserved molecular patterns shared by large groups of microorganisms. The dsRNAs represent one of these molecular patterns. Indeed, they are the key intermediates in the genomic replication of many viruses, but they are normally not found in eukaryotic cells. For this reason all higher eukaryotes respond to the presence of dsRNA with the activation of a range of native immune responses. In mammalian cells the major response is directed by the activation of the interferon pathway, that can block virus spread by inhibiting viral gene expression, causing apoptosis of infected cells and blocking the spread of the virus to neighbouring cells. In contrast to mammalian cells many other higher eukaryotes such as plants, nematodes and insects, do not base their antiviral response on the induction of interferon, but they are able of exerting another type of defensive response called RNA interference (RNAi) in animals and post translational gene silencing (PTGS) in plants. This response is based on the capability of an organism of recognising dsRNAs and degrading each mRNA that contains sequences identical to those of the dsRNA. The key characteristic of RNAi is its remarkable sequence specificity. Indeed, only mRNAs that have sequences of 19-20 nucleotides identical to those of the viral dsRNA are degraded. However also in mammalian cells the system has been found to be present, even if it is not yet clear if it has a role in virus defence.

For many years it was believed that this system was absent in mammalian cells, but on 2001 a paper by Elbashir and colleagues showed that, once inserted in mammalian cells, short dsRNAs of 19-21 nucleotides (called short interfering RNAs: siRNAs), that are usually unable of inducing interferon response, they could induce the RNAi system and cause a very strong degradation of mRNAs containing a sequence identical to the one of these siRNAs (74). The technique is very powerful since it allows to target virtually any mRNA opening the possibility of blocking the production of any protein just by insertion of a siRNA. A huge amount of studies since 2001 used this technique, that has become in a few year a routinely used method to easily knock down the expression of a target protein.

Both biochemical and genetic approaches have led to the current model of the RNAi mechanism. The RNAi includes an initiation step followed by an effector one.

In the initiation step when a long dsRNA enters a cell, it is cut in small dsRNAs of 21-23 nucleotides by the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases. Dicer cleaves in an ATP dependent manner the entering dsRNA forming

siRNAs 21-23 nucleotides long characterised by a two nucleotides overhang at the 3', a 5' monophosphate and a 3' hydroxyl group. Dicer contains an RNA helicase domain, a domain of unknown function, a PAZ domain, two RIII domains and a dsRNA-binding domain.

In the effector step the formed siRNAs are incorporated in the RNA Induced Silencing Complex (RISC) in a process that is mediated by Dicer and by the Dcr-2-associated protein R2D2. The phosphorylation at the 5' is necessary for the siRNA to enter RISC. Upon its binding in this complex the siRNA is unwound in an ATP-dependent manner by an helicase domain that may be associated with RISC. The active RISC targets the homologous transcript by base pairing interactions and cleaves the mRNA between the 10^{th} and the 11^{th} nucleotide from the 5' terminus of the siRNA. Apart from the protein described, many other components are present in the RNA interference machinery such as the members of the Argonaute proteins, a very conserved family of proteins that interacts with RISC, and the eukaryotic translation initiation factor 2C2 (eIF2C2). However many details concerning RNAi remain to be elucidated.

When siRNA are used to knock down a target mRNA, siRNAs are directly transfected in cells and loaded on the RISC complex, skipping the initiation step.

Figure 10 schematically describes the effector step of the RNA interference for synthetic siRNAs.

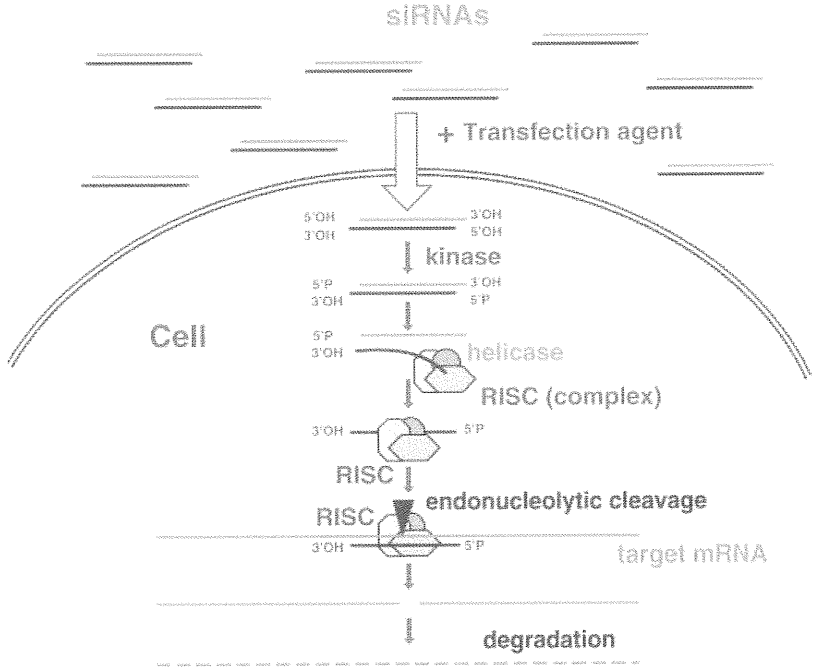


Figure 10: A schematic model of gene silencing by synthetic siRNAs. Once transfected into cells, synthetic siRNAs are phosphorylated, and a helicase, which may be associated with the RISC, leads to siRNA unwinding. The antisense strand of siRNA guides the complex towards the cognate mRNA. The target RNA is cleaved by an endoribonuclease in the RISC in a homology-dependent manner, resulting in mRNA degradation (50)

In comparison with other conventional drugs RNAi has many advantages: the system is sequence specific and this makes easy and flexible the selection of a target site; second, only substoichiometric amounts of siRNA are necessary for drastically decreasing target mRNA levels. Third, the length and homology required for siRNAs to work excludes, generally, the possibility of off targets effects. Indeed, siRNAs without a suitable homologous mRNA remain inert within the cell also suggesting that they do not affect adversely the cell; fourth, they can stably silence a target protein (228).

Although potentially any RNA can be the target of RNAi machinery it was not clear whether viral RNAs would also be effectively targeted. The reason for this is twofold. RNA virus genomes are often protected by a proteinaceous structure or through the association with cellular membranes during replication and so it may be inaccessible. This is for example the case of rotavirus, where the fact of having the dsRNA genome encapsidated in the core shell and never free in the cytoplasm protects it from being recognised by the interferon system or, possibly, by the RNAi machinery. In this case the only target of RNAi can be represented by viral mRNAs that are released in the cytoplasm of the infected cell. In addition studies made on plant and insect viruses had shown that they have evolved systems to suppress the RNAi machinery and avoid, in this way, the induction of a siRNA mediated immune response. Furthermore, very recently a similar type of response has been identified also in HIV-1 virus (17), suggesting that likely many viruses encode some kind of RNAi suppressor. However very often viral RNAi suppressor proteins block a step upstream of the generation of siRNAs and for this reason the use of chemical synthesised siRNAs can still be efficient in blocking production of viral proteins.

However RNAi has been used to target numerous viruses from diverse virus families and profound inhibition of virus replication both *in vivo* and *in vitro* has been obtained. This is particularly important for viruses (i.e. rotavirus) where reverse genetic techniques are not available and protein function had been studied with biochemical methods or through the use of temperature sensitive mutants. In addition concomitantly with this kind of functional studies, a series of studies regarding the possibility of using siRNAs *in vivo* for therapeutic purposes have been described (104, 228).

Positive stranded ssRNA viruses are an attractive target of RNAi since their genomes act as both mRNA and replicon template. Dengue virus represents one of the first animal viruses that could be inhibited by RNAi. It is a mosquito-borne virus of the family of *Flaviviridae* that can cause hemorrhagic fever in humans. The DEN genome is encoded by a (+) ssRNA of

11kb and generates detectable levels of dsRNA as an intermediate of its replication. Already in 2001 Adelman and colleagues showed that the expression of an inverted repeat RNA derived from the genome of the virus caused a very strong (more than 95%) decrease in dengue antigen accumulation. In this type of approach the target of siRNAs was Dengue replication in *Aedes aegypti* mosquitoes, the insect responsible of transmission of the virus to human (2, 3). However positive results were obtained as well with siRNAs targeting the 3' non coding sequence of the virus common to all dengue serotypes in Vero or DC cells (261).

Human hepatitis virus (HCV) is a major cause of chronic hepatitis and hepatocellular carcinoma. Like dengue virus it is a member of the *Flaviviridae* and contains a (+) ss RNA genome. Given the medical importance of HCV infections, several laboratories have investigated the possibility of inhibiting the replication of the virus and demonstrated that many viral sequences can be targeted with RNAi causing a drastic reduction of the virus infection. Among these sequences there were those coding for the viral polymerase NS5B or the non-structural protein NS5A (160, 218). In addition also the well conserved 5' non-coding region of the virus, where the internal ribosomal entry site (IRES) necessary for translation is present, has been targeted with a strong inhibition of virus replication (219).

Among the (-) ss RNA viruses influenza virus represents one of the major human pathogenic viruses causing severe respiratory infections. Ge and colleagues targeted several viral sequences conserved among different subtypes and strains of influenza virus A and proved that those targeting nucleocapsid and RNA polymerase efficiently inhibited influenza virus production both in vitro and in chicken embryos (88, 111). Interestingly, for therapeutic purposes it has been proposed to administer siRNAs via intranasal or pulmonary routes as it has been recently made with success for the human respiratory syncytial virus and parainfluenza virus, two other (-) strand viruses of the family of *Paramyxoviridae* (21).

Also DNA viruses have been shown to be suitable targets for RNAi, even if in this case the target is viral mRNAs produced after infection. An example is represented by human papilloma virus (HPV). These are small viruses with a genome of approximately 8kb. HPV16 is the main causative agent of cervical cancer and encodes E6 and E7, that are two oncogenes necessary for the malignant transformation. siRNAs against E6 showed to strongly inhibit E6 protein production and exhibited a potent growth inhibitory activity (254). Silencing of E7 induced selective loss of the hyperphosphorylated form of Rb and caused cells to undergo apoptosis (116).

With respect to retroviruses, many studies have targeted HIV with the RNAi technique also in search for alternative strategies of treatment. Many HIV-1 genes have been successfully targeted such as the structural proteins Gag and Env, the polymerase Pol, the regulatory proteins Tat and Rev and the accessory proteins Nef and Vif. In addition also the long terminal repeats of the genome have been effectively targeted (104).

Among the dsRNA viruses rotavirus is the only one that has so far been targeted with RNAi. Many rotavirus proteins have been inhibited by siRNAs with different outcomes, as it has been described previously in this thesis.

Even if RNAi has proven to be very efficient in targeting viral proteins and blocking viral replication, viruses characterised by a high rate of mutation of their genome have been demonstrated to be able to escape RNAi through point mutations in the region of homology targeted by the siRNA. This is the case for example of Poliovirus, where siRNAs were able of inhibiting virus production 100-fold, but the virus titer increased upon prolonged incubation. Sequencing of the escaped viruses showed the presence of point mutations in the region of homology with the siRNA (89). Similar results have been obtained also for HIV-1 (24, 57). For therapeutic purposes the targeting of multiple sequences, in particular the well conserved ones, should reduce the probability of generating escape mutants.

Another possible use of RNAi can be the inhibition of cellular partners of viral proteins both for research and for therapeutic purposes. Receptors for virus binding and entry are attractive for therapeutic purposes, because their absence leads to the block of all subsequent steps of viral cycle and in addition cellular targets are unlikely to generate escape mutants. An example is represented by CCR5, the chemokine co-receptor for the majority of HIV-1 isolates. RNAi against CCR5 protected cells against HIV-1 infection (139). Another example is represented by the glucose transporter GLUT-1, that has been demonstrated to be a component of the receptor for the human T cell leukaemia virus targeting the 3' non-coding region of its mRNA (154). However also intracellular targets have been targeted by RNAi in order to assess their role in viral replication. Silencing of the human helicase p68 for example caused a decrease in the transcription of negative strand HCV RNA (90).

In general RNAi proved to be very efficient in blocking virus protein synthesis in many types of viruses including RNA viruses with linear or fragmented genomes of positive or negative polarity as well as DNA viruses and have helped to understand many aspects of viral replicative cycles.

In order to evaluate the role of NSP5 in the replicative cycle of rotavirus, we used the RNA interference approach.

We targeted NSP5 of two different strains of rotavirus with siRNAs specific for each one of them and evaluated how the lack of NSP5 was affecting the viral protein production, viroplasm formation and virus replication.

In addition we targeted, through the use of a specific siRNA, CK1α, the kinase that during in vitro experiments had been shown to be responsible for phosphorylation of NSP5 in serine 67. We evaluated if phosphorylation of serine 67 was occurring also in vivo and we investigated the effect of the decreased phosphorylation of NSP5 on the replicative cycle of rotavirus.

MATERIALS AND METHODS

Cell culture

MA104 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) (Gibco-BRL), 2mM L-glutamine and gentamicin (50 μg/ml) (complete medium).

Stable transfected cells (C7 and NSP2-EGFP) were cultured in complete medium supplemented with geneticin (G-418) 500 μg/ml (Gibco-BRL) as previously described (6, 72).

Cells were propagated using trypsin (500 μg/ml) in order to detach them from the plate, centrifuged 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 (79). Briefly, the inoculum was activated in presence of 10μg/ml of trypsin for 30 min at 37°C and let to absorb on the cell monolayer for 1 h at 37°C. Cells were thus washed and cultured in serum free medium with 10μg/ml trypsin until complete cytopathic effect was visualised. The infective medium was frozen and thawed three times, centrifuged at 5000 rpm for 10 min to eliminate cellular debris and stored at -80°C.

Vaccinia strain vTF7.3, recombinant for the T_7 RNA polymerase gene, was propagated in HeLa cells as described by (84).

Small interfering (si) RNAs and transfection

The siRNAs were chemically synthesized with a 3' TT deoxynucleotide overhang on both strands, and obtained as annealed duplexes (IBA). A fluoresceinated siRNA against SA11 segment 11 (si/SA11-fluo) with 6-FAM on the 5' of the sense strand was obtained as annealed duplex (Quiagen). Approximately 1.5 x 10^5 cells were transfected with 2 μg of siRNAs in 1 ml of serum free medium containing 5 μl Transfectam reagent (Promega). After 6h at 37 °C, cells were washed twice with serum free medium and incubated for additional 34-72 h in medium supplemented with 10% fetal bovine serum (Gibco).

Transient transfection of MA104 cells with vaccinia virus

Vaccinia virus is used to obtain an efficient transient expression of genes under the control of T_7 promoter. Since vaccinia virus cycle is cytoplasmatic, exogenous gene transcription and translation are coupled in the cytoplasm of the transfected cells.

Cells were transfected with siRNAs, as previously described, and, 32 h after, infected with vaccinia virus at a multiplicity of 20 pfu/cell in serum free. One hour later, the cells were washed and transfected with 5μl of Transfectam reagent containing 2μg of plasmid (or 1+1 in the case of cotransfection of two plasmids) and incubated for 16h.

VP1 cloning and production of anti VP1 antibody

VP1 sequence was cloned by SA11 infected cells. Viral RNA was extracted from 500μl of virus inoculum through phenol-chlorophorm treatment and ethanol precipitation. cDNA was obtained through RT-PCR using random examers, and MuLV reverse transcriptase (Applied Biosystem). Subsequently, VP1 was amplified in two pieces through PCR: VP1/I comprises from nucleotide 19 (corresponding to the start codon) to nucleotide 1322 of the genomic segment 1 and VP1/II comprises the rest of the protein from nucleotide 1231 to the stop codon at nucleotide 3285. In the sequence in common between the two amplified fragments (nucleotides 1231 to1322) is present an NsiI site that has been used in the subsequent stages to join the two VP1 fragments to form the total protein.

The primers used to amplify VP1 were:

VP1/I for: 5'TAGGGTACCATGGGGAAGTACAATCTAATC3'

VP1/I rev: 5'ATGGAATTCGGCGTGTATCTTTCGTTAGC3'

VP1/II for: 5'TAGGGTACCTCAAGGCAGCTAAAGTTTGG3'

VP1/II rev: 5'CCGCTCGAGCTAATCTTGAAAGAAGTTCGC3'

Fragments were cloned in pGEM vector (Promega), sequenced and subsequently subcloned in Pet23d vector (Novagen) containing an histidine tag to express the protein in bacteria and in pcDNA3 (Invitrogen) for expression in mammalian cells.

Sera were obtained through protein immunisation using Histidine tagged VP1/I. His tagged protein was produced in *E. coli* Bl21 strain. Cultures were induced with 3mM IPTG for 3-4 h at 37°C. The bacteria were centrifuged and the pellet washed with ice cold PBS and resuspended in 1,5% laurilsarcosine-PBS and added 0,1 μg/μl lysozime, 0,1 μg/μl

CLAP(protease inhibitor mix), 5 mM DTT for sonication (6 times 10s). The supernatant was supplemented with 1% Triton X-100 in PBS and with Ni-NTA His-Bind resin (Novagen) equilibrated in 20mM imidazole in PBS. After rolling for 1h at 4°C, the sample was centrifuged at 1000xg for 5 min at 4°C and the resin was washed with 10 volumes of imidazole 35mM in PBS. Elution was performed with two volumes of imidazole 250mM.

Balb/c mice and guinea pigs were injected intraperitoneally with 50µg of protein or 100µg for each boost respectively. The boosts were repeated three times every 15 days and the animals bleeded one week later. From each blood sample was recovered about 200µl of sera from the mice and 500µl from guinea pigs. Antibodies were tested by Western Immunoblot analysis on extracts of infected and uninfected cells.

Antibodies

Sera against NSP5, NSP2, VP7 were produced by immunisation of guinea pigs and mice with GST fusion proteins essentially as described (95). Dilutions used for primary and secondary antibodies used in western blot and immunofluorescence are listed in tables 2 and 3 respectively.

Table 2. Antibodies for WB and dilutions

Primary 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)
Guinea pig anti-VP1 (1:1000)	Goat anti-guinea pig-HRP (1:2500)(KPL)
Mab anti-SV5 (1:5000)	Goat anti-mouse-HRP (1:5000)(KPL)
Rabbit anti-VP7 (1:1000)	Goat anti-rabbit-HRP(1:5000)(KPL)
Rabbit anti-actin(1:400) (Sigma)	Goat anti-rabbit-HRP(1:5000)(KPL)

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

Table 3. Antibodies for immunofluorescence and dilutions

Primary antibody	Secondary antibody[a]
Guinea pig anti-NSP5 (1:300)	Goat anti-guinea pig-RITC or -FITC (1:100) (KPL)
Guinea pig anti-NSP2 (1:100)	Goat anti-guinea pig-RITC or -FITC (1:100) (KPL)
Mouse anti-NSP5 (1:100)	Goat anti-mouse RITC or FITC (1:100) (KPL)
Mouse anti-NSP2 (1:100)	Goat anti-mouse-RITC or FITC (1:100) (KPL)

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

Cellular lysis

Lysates (corresponding to about $5x10^5$ cells) were prepared in 100µl of TNN lysis buffer (100mM Tris-HCl pH8.0, 250mM NaCl, 0.5%NP40, 1mM PMSF) for 10 minutes at 4°C. Lysates were subsequently centrifuged at 10000xg for 5 min to eliminate cell nuclei and membranes. Usually 1/5 of the extract was loaded in SDS-PAGE for Western immunoblot and 1/2 was used for immunoprecipitation.

Immunoprecipitation

Cellular extracts were immunoprecipitated 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 or O.N. at 4°C. Beads were washed three times with TNN buffer and samples analysed in SDS-PAGE (131).

In vivo [^{35}S]-methionine labelling and DSP crosslinking

MA104 cells were transfected as previously described and 48h after infected with rotavirus. After 4h from infection cells were starved in DMEM lacking methionine for 30 min and then 300µCi of [^{35}S] methionine was added for 30 min. the cells were washed in 2 times PBS, incubated 10 min in 25 mM DSP (Dithiobis(succinimidyl propionate),Pierce) at 4°C. Cells were washed 3 times in 2,5 ml TBS (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)) for 10 min at 4°C and centrifuged at 10,000 x g for 5 minutes. 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 (131). 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).

λ phosphatase treatment of cellular extract.

5 μl of a cellular extract obtained from the transfection/infection of about $5x10^5$ cells were incubated with 4 μl (400 U/μl) λ-phosphatase, buffer λ-phosphatase (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM DTT, 0.01%) and 1X $MnCl_2$. The reaction was incubated for 2 h at 30°C. The reaction was stopped with 5 μl of loading buffer.

Western blot

The cellular extracts were resolved in a SDS-PAGE (131). After electrophoresis samples were transferred to a PDVF membrane (Immobilion-P), for 2 h at 200 mA or over night at 50 mA. The membrane was blocked in PBS-milk 5% for 30 min and incubated for 1 hr in PBS-milk 5% with the primary antibody. After three washes in PBS-milk 5%, the secondary antibody conjugated to horse radish peroxidase (HRP) was added and incubated for 1 h. Primary and secondary antibody concentrations were those indicated in table 2. 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).

Indirect immunofluorescence

For indirect immunofluorescence microscopy cells were fixed in 3,7% paraformaldehyde in PBS for 10 min at room temperature. Cover slips were washed in PBS and blocked with 1% BSA in PBS for 30 min and incubated with primary antibody at the concentration shown in table 3 for 1 h in moist chamber at room temperature. After three washing in PBS, slides were incubated either with another primary antibody for double staining or directly for 45 min with RITC- or FITC-conjugated secondary antibodies. After three washings nuclei were stained with were incubated with Hoechst dye 2μgr/ml for 10 min, washed and mounted with ProLong mounting medium (Molecular Probes). Samples were analysed by confocal microscopy (Axiovert; Carl Zeiss) or with cool SNAPs system using a fluorescence microscope (DMLB,Leica). Around 500 cells were counted per each experimental point and the experiment repeated at least three times. Viroplasm positive cells were considered those with two or more dots per cell.

Viral genomic dsRNA

Equal numbers of rotavirus-infected cells were collected and total genomic dsRNA was prepared as previously described (41). Briefly: cells transfected and infected for 16hrs were lysed in sterile TNN buffer for 10 min at 4°C. RNA was subsequently extracted with TNN buffer saturated Phenol. The aqueous phase was precipitated with 4 volumes of ethanol. RNA pellet was subsequently Resuspended in sterile water sample buffer and samples were electrophoresed in a 10% polyacrylamide gel, 0.75 mm thick, for 15 h at 20 mA and bands visualised with ethidium bromide.

RESULTS (1)

The fist aim of the first part of this work was to assess the effect of the lack of the non structural protein NSP5 in the context of viral infection through the use of RNA interference.

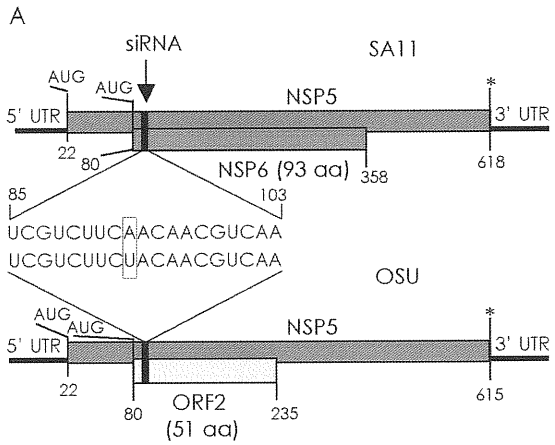
Construction of the siRNAs

We constructed two double stranded siRNAs of 19bp to target rotavirus gs11 mRNA between the nucleotides 85 and 103 within the NSP5 and NSP6 coding region in two different rotavirus strains, the simian strain SA11 and the porcine one OSU. As mentioned in the introduction (as described in § 1.4.6.), the gs11 of OSU does not code for a full length NSP6 but for a truncated form that reduces the coding region to only 51 residues (235) and that, for this reason, will be called, ORF2. The target sequences were chosen based on general rules suggested for siRNAs design:

- localisation around 50 to 100 nucleotides downstream of the start codon
- choice of a region outside of 5' or 3' UTRs sequences
- 50% G/C content
- TT deoxinucleotide overhang at the 3'

Figure 11A schematically describes the SA11 and OSU gs11 mRNA showing the two ORFs corresponding to NSP5 and to NSP6 (or ORF2 in OSU). In addition the region targeted by the siRNAs are indicated. It is worth noting that the two regions targeted, and as a consequence also the si/RNA are identical except that for a single nucleotide localised at position 93.

The two synthetic siRNAs for SA11 and OSU were termed respectively si/SA11 and si/OSU and are shown in figure 11B.



B

si/SA11
5′ UCGUCUUCAACAACGUCAATT 3′
3′ TTAGCAGAAGUUGUUGCAGUU 5′

si/OSU
5′ UCGUCUUCUACAACGUCAATT 3′
3′ TTAGCAGAAGAUGUUGCAGUU 5′

Figure11: (**A**) Diagram of SA11 and OSU genomic segment 11 mRNA. The ORFs corresponding to NSP5 and NSP6 (in SA11) or ORF2 (in OSU), are indicated. The regions targeted with siRNAs are shown expanded with the only nucleotide difference boxed. UTR, untranslated regions; AUG, initiation codons. Numbers correspond to nucleotide positions. (**B**) Sequence of the synthetic double stranded siRNAs specific for rotavirus strains SA11 (si/SA11) and OSU (si/OSU) covering nucleotides 85-103 of the corresponding gs11 mRNAs.

Efficiency of NSP5 transfection and effect on its production

In order to evaluate the activity of these siRNAs, we transfected them in MA104 cells, a cell line widely used in rotavirus experiments because easily infectable. After 72 hours from siRNAs transfection, the cells were infected with either SA11 or OSU virus for 5 h. The efficiency of transfection was about 80% as it could be evaluated through the use of a fluoresceinated si/SA11 (see figure 16).

In figure 12A it is possible to see the effect of the two siRNAs on OSU and SA11 NSP5. As described in the introduction (as described in § 1.4.5.), NSP5 derived from infected cells migrates in SDS-PAGE as two major bands of 26 and 28 kDa and a series of higher bands whose molecular weight span from 30 to 34kDa that derive from processes of phosphorylation of the protein. As shown in the figure 12A a large reduction in the amount of

SA11 or OSU NSP5 could be observed in cells transfected with the corresponding siRNA. In addition there was practically no effect when cells were infected with either virus that had received the non-homologous siRNA as well as in the case of the control protein actin. These results indicate that the siRNAs were very efficient in blocking the production of NSP5 and that their action was extremely specific, since each siRNA was able of blocking only the production of NSP5 coming from the homologous virus and no effect could be noticed in a non relevant protein used as control (actin).

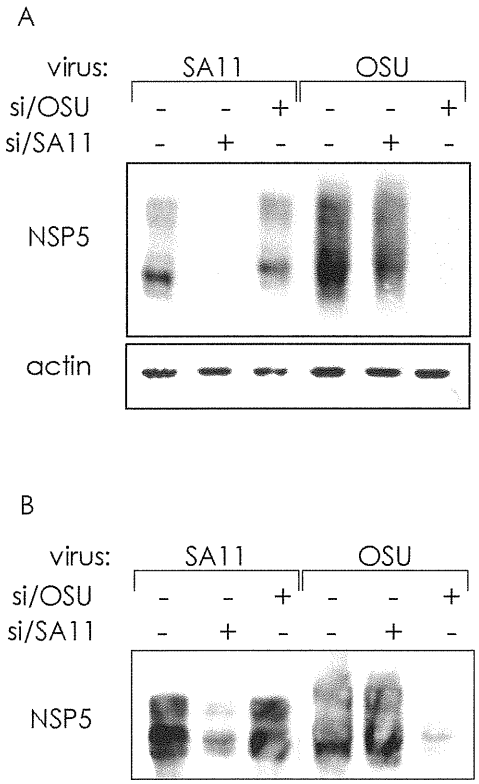


Figure 12: (A) Western blot analysis of viral proteins NSP5 and cellular actin at 5 h p.i. on total extracts of virus-infected MA104 cells untreated (-) and treated (+) with si/SA11 or si/OSU, as indicated. **(B)** Western blot analysis of NSP5 expression of virus-infected MA104 cells at 15h p.i. in si/SA11 or si/OSU treated cells, as indicated.

At longer times of infection (15h), a significantly larger amount of NSP5 is observed, probably due to cells that did not uptake the siRNA or to the fact that continuous production of viral mRNAs was able to override the interfering activity (Figure 12B).

Effect of RNA interference on gs11 on the production of other viral proteins

In order to understand if the lack of NSP5 could have a role in the production of other viral proteins, extracts of cells treated with siRNAs and virus infected were tested with sera against both viral structural and non structural proteins such as VP1, NSP2, and VP7. As shown in figure 13, the lack of NSP5 correlated with a block in production of all the viral proteins tested. It is of interest to notice that are inhibited both proteins that are localised in viroplasms such as the viral polymerase VP1 and NSP2 and proteins that are localised in other compartments of the cell and have no relation with the viroplasms such as VP7 (ER localised), suggesting that in absence of NSP5, there is a generalised inhibition on the production of viral proteins. We produced the antibody against the viral polymerase VP1 immunising animals with a VP1 fragment derived from the SA11 sequence and, for this reason, the OSU form is not recognised.

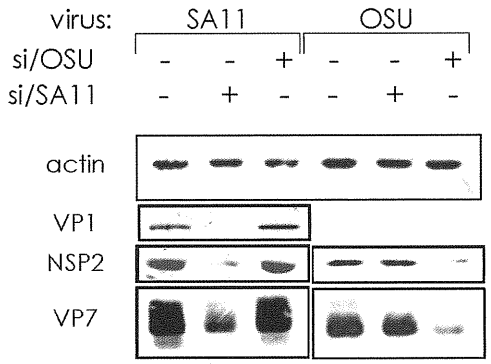


Figure13: Western blot analysis of structural and non structural viral NSP2, VP1 and VP7 and cellular actin at 5 h p.i. on total extracts of virus-infected MA104 cells untreated (-) and treated (+) with si/SA11 or si/OSU, as indicated.

Assessment of viroplasm formation in siRNA transfected cells

As described in the introduction, viroplasms are electrondense structure that can be found in the cytoplasm of rotavirus infected cells where virus replication and packaging occur.

Since NSP5 localizes in cytoplasmic viroplasms, we analysed their formation in cells treated with siRNAs. The cells were siRNA transfected for 72 h and virus infected for 5 hrs and afterwards fixed with paraformaldehyde and visualised by immunofluorescence. Since also NSP2 localises in viroplasms and is in tight association with NSP5 (5), we used an antibody anti-NSP2 to visualise them. As it can be seen in the representative images of figure

14, the knocking down of NSP5 expression heavily impaired the formation of viroplasms. Once more the effect showed to be highly sequence specific since si/SA11 and si/OSU strongly inhibited the formation of viroplasms in cells infected, respectively, with SA11 (75-80% inhibition) and OSU (80-83%) inhibition. As for the production of viral proteins, the cells infected with the non homologous strains were fully competent in sustaining viroplasm assembly. Moreover the few viroplasms remaining in siRNA-treated cells appeared smaller and reduced in number.

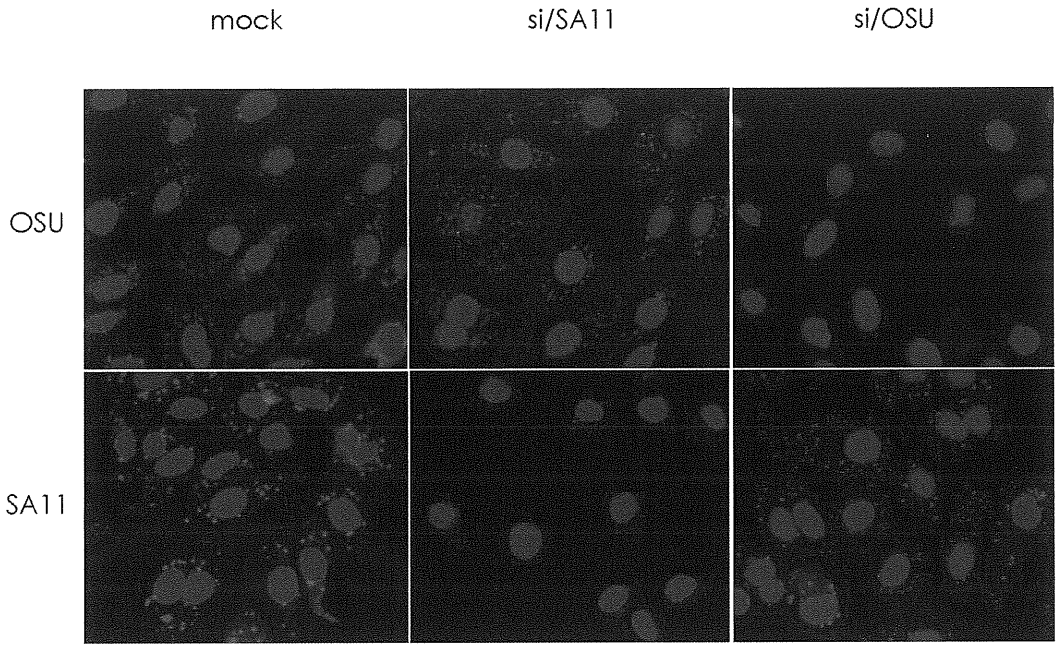


Figure 14: Viroplasms in siRNA-treated cells. Immunofluorescence of MA104 cells mock-transfected or transfected with si/SA11 or si/OSU and infected with the indicated virus strain. Viroplasm were visualised with an anti-NSP2 antibody (red). Nuclei stained with Hoechst dye (blue).

Additional information on the process were obtained through the use of a cell line, called C7 (5), that stably expresses an OSU-NSP5 that is, therefore, resistant to si/SA11. As shown in figure 15, this cell line was able of partially rescue the formation of viroplasms for the lack of SA11-NSP5 in cells treated with si/SA11. The fact that a cell line expressing an OSU-NSP5 is able of rescuing the formation of viroplasms demonstrates that the phenotype observed is not due to the lack of NSP6 since the sequence of OSU-ORF2 is a truncated form of NSP6 accounting for around half of the ORF of the wild type protein.

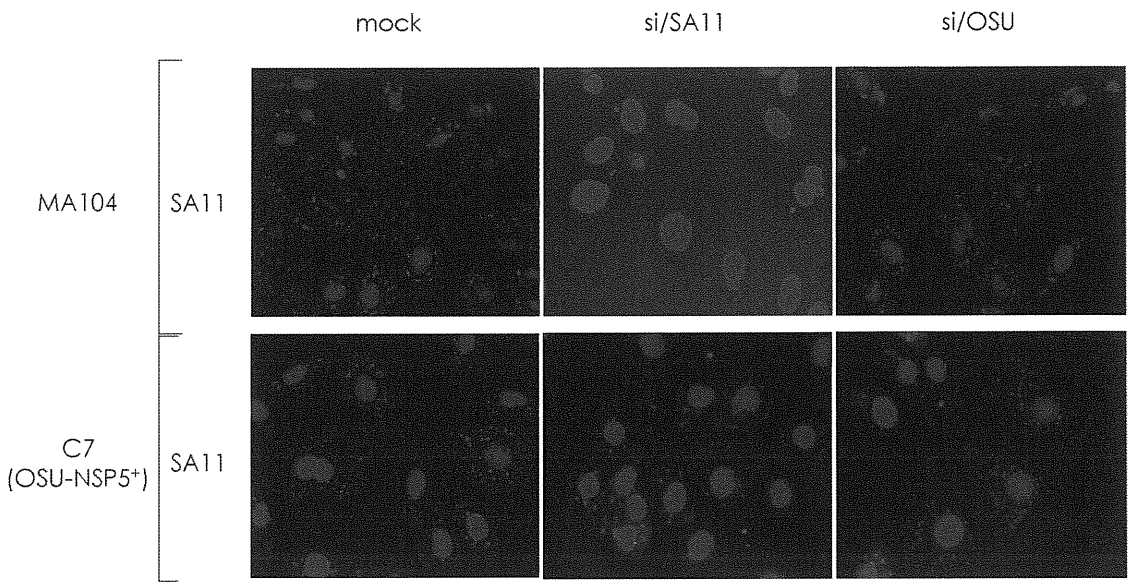


Figure 15: Viroplasms in siRNA-treated cells. Immunofluorescence of MA104 or C7 cells mock-transfected or transfected with si/SA11 or si/OSU and infected with the indicated virus strain. Viroplasm were visualised with an anti-NSP2 antibody (red). Nuclei stained with Hoechst dye (blue).

We used a fluoresceinated si/SA11 in order to visualise the presence of siRNAs in the cell and to estimate the percentage of transfected cells. We calculated that more than 80% of cells were transfected and unable to form viroplasms after SA11 infection, while infection with OSU was unaffected (Figure 16). In addition it could be visualised that si/SA11-fluo localised as punctuate foci in a cytoplasmatic region adjacent to the nucleus, a localisation characteristic of siRNAs in general (121).

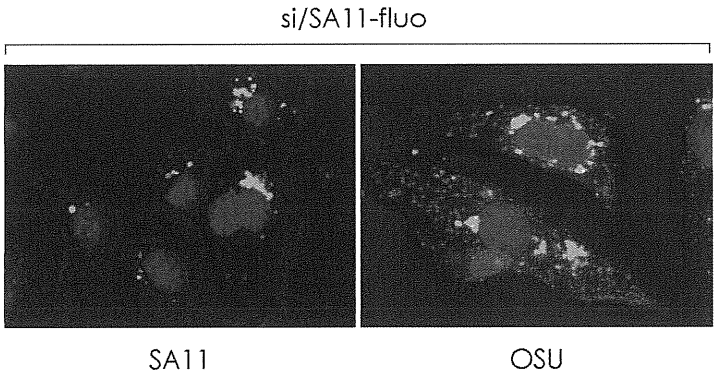


Figure 16: MA104 cells transfected with fluoresceinated si/SA11 (si/SA11-fluo, green) and infected with the indicated virus strain. Viroplasm were visualised with an anti-NSP2 antibody (red). Nuclei stained with Hoechst dye (blue).

Use of intrabodies specific for rotavirus NSP5 to evaluate its role in the viroplasms formation

A further confirmation of the importance of NSP5 in the formation of viroplasms has been obtained in a study made in parallel with the data presented so far and to which I participated. It is a protein knock down system based on the use of specific intracellular antibodies (ICAbs) (241). In this study, a library of scFv was obtained by NSP5 immunised mice and selected through the yeast two-hybrid system for ICAbs specific for NSP5. Two of these ICAbs (called A19 and D24) demonstrated to specifically recognise NSP5 both in transfected and in virus infected cells (240). In order to visualise the expression of the ICAbs in infected cells, they were expressed as fusion proteins with EGFP. A not relevant ICAb (3b) and EGFP alone were used as controls. ICAbs were transfected into MA104 cells and, 30 hrs after transfection, cells were infected with rotavirus strain OSU for 5 or 8 hrs. Cells were fixed and stained with anti NSP2 or anti NSP5 antibody. Since not all cells were transfected the status of rotavirus infectivity was analysed only in cells expressing ICAbs-EGFP. We found a remarkable difference in the number of viroplasms positive cells in cells transfected with A19-EGFP or D24-EGFP compared with controls. As shown in figure 17 the inhibition of viroplasm formation was specific for the two ICAbs (A19 and D24), as neither 3b-EGFP nor EGFP alone had any significant effect on the formation of viroplasms at both times analysed even if the inhibition was stronger for shorter times of infection. Since the ICAbs are specific for NSP5 and not target NSP6 this result is a further indication of the fact that NSP5 is the protein responsible of the effect on viroplasm formation that we observe.

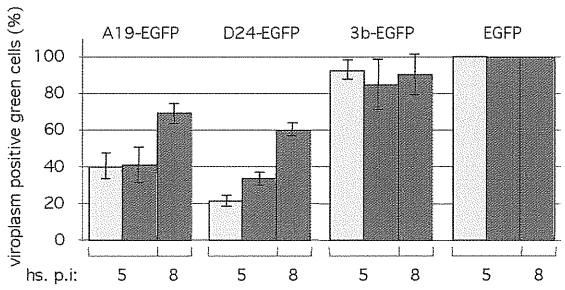


Figure 17: Percentage of viroplasms positive cells transfected with the various constructs as indicated and subsequently infected with rotavirus for 5 or 8 hours as indicated. Viroplasms were detected with either anti-NSP2 antibody (dark blue) or anti-NSP5 antibody (light blue). The number of viroplasm-positive cells transfected with EGFP was considered to be 100%.

Effect of siRNA treatment on the cytopathic effect of rotavirus

In the MA104 cells, the outcome of rotavirus infection, after 12-18 hrs from infection, is a cytopathic effect that causes the complete lysis of infected cells and their detachment from the culture plate. In order to evaluate if there was an effect of the lack of NSP5 in this process, cells were siRNA transfected as previously mentioned, and infected for 15 hrs Figure 18 shows representative images of the cytopathic effect observed: treatment of cells with si/SA11 and infection with the corresponding virus, SA11, caused a very strong decrease in the cytopathic effect, indeed these cells were practically devoid of lytic effect and resembled the control, mock-transfected not-infected cells. As expected, si/OSU treated cells show the same phenotype of mock-transfected SA11-infected cells.

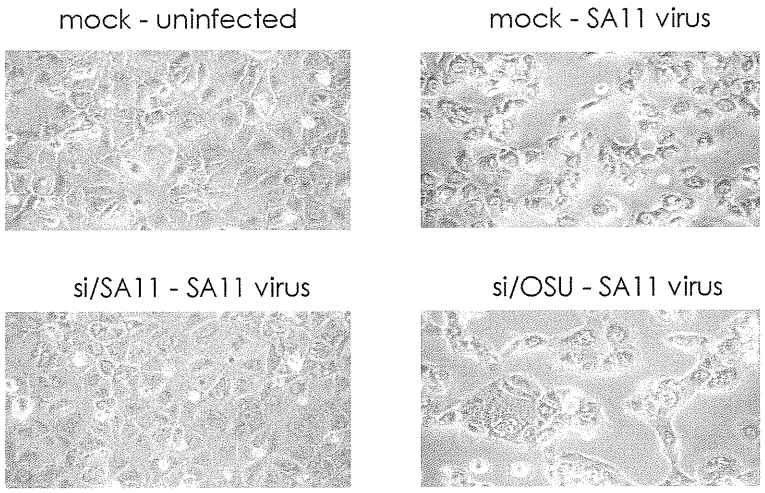


Figure 18: Cytopathic effect on si/SA11-treated (bottom left panel) or si/OSU-treated (bottom right panel) cells at 15 h post-infection with virus strain SA11 compared to mock-transfected uninfected cells (upper left panel) and to mock-transfected SA11-infected cells (upper right panel).

Effect of RNA interference on virus production

The effectiveness of si/RNA treatment on viroplasms formation and on viral cytopathic effect, suggested an impaired virus replication. In order to investigate further this aspect, we analysed the production of infective virions by siRNA-treated virus-infected cells. Virus inocula coming from cells either mock transfected, si/SA11 transfected or si/OSU transfected and SA11 infected for 16 hrs were used to infect new cells. Two types of cell lines were infected with these virus inocula: wild type MA104 cells and a stable transfectant cell line expressing NSP2-EGFP fusion protein that shows, upon virus infection, relocalisation of the cytoplasmic diffused protein to viroplasms (72). This is a very convenient cell line since, the formation of viroplasms can be visualised directly without the use of antibodies.

As shown in representative figure 19 upper row, the immunofluorescence of MA104 cells using an anti-NSP2 antibody, showed that si/SA11 but not si/OSU inhibited the production of infective SA11 virus. Similar results were obtained through the use of the stable cell line NSP2-EGFP (Figure 19 lower row).

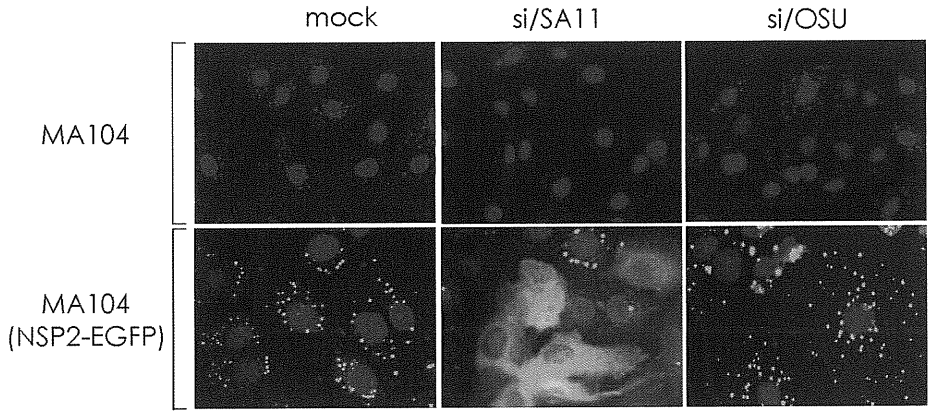


Figure 19: Production of infectious virions in siRNA-treated cells. SA11 infective particles produced by untreated, si/SA11-treated or si/OSU-treated cells and detected by their ability to produced viroplasms upon infection of MA104 (upper row, anti-NSP2 red) or MA104 cells stably expressing NSP2-EGFP fusion protein (lower row, green). Nuclei stained with Hoechst dye (blue).

In order to be able of quantify the inhibition of virus production, we determined virus titers. As shown in table 2, the virus titers were reduced between 10 and 20 fold depending on the virus strain in cells treated with the corresponding homologous si/RNA. It is worth noting that, as previously mentioned, not all cells can be transfected with si/RNA, and for this reason a number of them will sustain virus replication. In different experiments we observed that the reduction in OSU titers was always higher than that obtained with SA11, consistent with what we observed by immunofluorescence.

Virus titers (ffu/ml)

		Experiment 1	Experiment 2	Experiment 3
SA11 infection	mock	$4.5x10^6$	$4.2x10^6$	$7.8x10^6$
	si/SA11	$3.5x10^5$	$3.8x10^5$	$5.6x10^5$
	Si/OSU	$3.6x10^6$	$3.9x10^6$	$6.2x10^6$
OSU infection	mock	$7x10^6$	$6x10^6$	$3.8x10^6$
	si/SA11	$5.5x10^6$	$4.8x10^6$	$3.5x10^6$
	si/OSU	$2.7x10^5$	$1.6x10^5$	$2.2x10^5$

Table 2:Virus titers were determined by immunofluorescence of MA104 infected cells with anti-NSP2 antibody

A final confirmation of the strong block of virus replication due to the lack of NSP5 has been obtained through the visualisation of SA11 and OSU dsRNA genome. As mentioned in the introduction, the rotavirus genome is composed by 11 segments that can be separated in an acrilamide gel where they display a typical pattern in which the various genomic fragments can be easily identified. In order to understand what was the outcome of siRNA treatment on the formation of the double stranded genomic segments, we transfected cells with either siRNA and infected them for 16 hrs. As shown in figure 20, SA11 dsRNA production was greatly inhibited by treatment with si/SA11 and the same held true for OSU virus. Furthermore, in agreement with what was found for viroplasm formation, complementation of SA11 dsRNA production was observed in C7 cells treated with si/SA11 and SA11 infected. As expected, C7 cells could not rescue for the virus replication in si/OSU transfected OSU infected cells, since in this case also the production of the 'cellular' OSU-NSP5 was blocked by the siRNA.

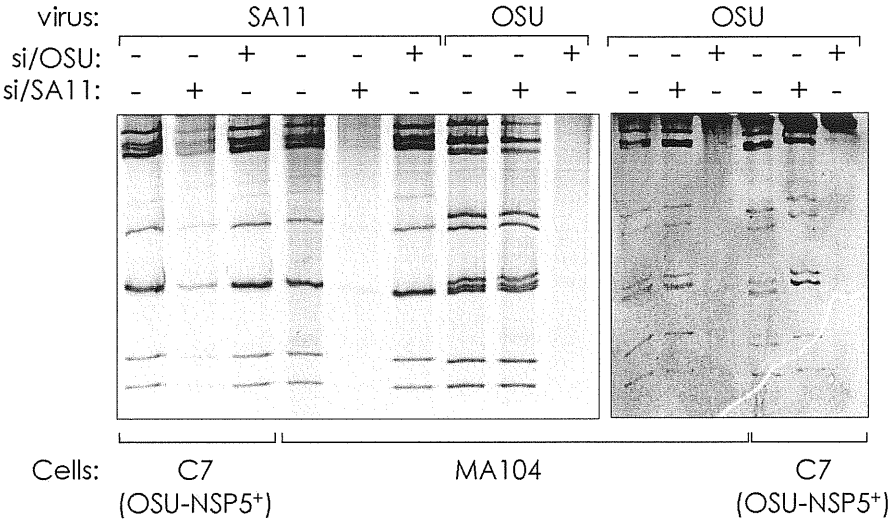


Figure 20: Viral genomic dsRNA production in MA104 or C7 cells mock-transfected (-) or transfected (+) with si/SA11 or si/OSU, and infected with either SA11 or OSU virus strains, as indicated.

Taken together, these results revealed the essential role of NSP5 in the assembly of viroplasms and in virus replication.

RESULTS (2)

Previous studies of our laboratory had shown that the process of hyperphosphorylation of NSP5 is rather complex and allowed to depict a model (figure 8) in which the interaction between NSP2 and NSP5, causes a conformational change in NSP5 and allows it to be phosphorylated in ser-67. This seems to be the first step in the process of NSP5 hyper-phosphorylation. As described in the introduction, these results were obtained through the use of a series of mutants of NSP5 in co-expression experiments in absence of viral infection. One of the main results was the indication that CK1α is the cellular kinase responsible of the phosphorylation of NSP5 in ser-67. This was obtained through *in vitro* phosphorylation assays in which NSP5 peptides or NSP5 mutants produced in bacteria, were incubated with a recombinant CK1α coming from zebrafish (71). In order to evaluate if the mechanism of NSP5 phosphorylation by CK1α obtained through *in vitro* experiments, was the same *in vivo* we decided to use an RNA interference approach.

Construction of the siRNA specific for CK1α and effect on the target protein

To assess if the lack of CK1α had an effect on NSP5 phosphorylation it was constructed a siRNAs specific for CK1α. This siRNAs was designed based on sequences identified in previous publications to be suitable for targeting the various isoforms of CK1 and was called si/CK1α (107, 143). Figure 21 schematically describes the synthetic siRNA constructed.

si/CK1α
5′ CCAGGCATCCCCAGTTGCTTT 3′
3′ TTGGTCCGTAGGGGTCAACGA 5′

Figure 21: Sequence of the synthetic double stranded siRNA specific of CK1α

It is important to note that this sequence is specific for CK1α and does not target other CK1's isoforms such as CK1δ or CK1ε.

Role of CK1α in phosphorylation of Δ2 by Δ3

As mentioned in the introduction, mutants of NSP5 that lack region 1, region 3 or both (figure 5), have the capability to act as activators of the phosphorylation of NSP5 or of its deletion mutant Δ2 (71). In order to evaluate the role of CK1α in the process, cells were co-transfected with Δ2 and Δ3 in presence of either an irrelevant siRNA (si/irr) or of si/CK1α. To identify Δ2 unequivocally, the mutant was tagged at the amino terminus with a SV5 tag and the protein detected through the use of a specific antibody against SV5. As it can be noticed in figure 22, as expected, when SV5-Δ2 is expressed alone it gives rise to a unique band and the absence of CK1α does not induce any change in the protein mobility shift (lanes 1 and 2). When SV5-Δ2 is co-expressed together with Δ3, the activator induces a strong phosphorylation of SV5-Δ2 with the appearance of three slow migrating bands (lane 3). Lack of CK1α has a strong effect on the phosphorylation of SV5-Δ2 by Δ3 suggesting that, in agreement with *in vitro* experiments, phosphorylation of Δ3 by CK1α is necessary for this mutant to act as activator also *in vivo*. However it can be noticed by comparing lane 4 with lane 1 or 2 that the block of phosphorylation of Δ2 is not complete, suggesting that other cellular kinases might be involved in the process or that the inhibition by the siRNA is not complete.

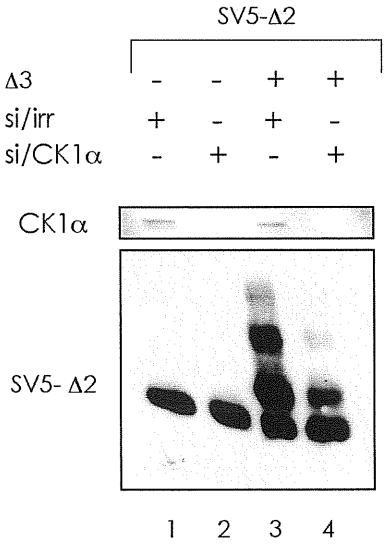


Figure 22: Coexpression of SV5-Δ2 mutant alone or with the activator Δ3 in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect SV5 and CK1α as indicated.

Role of CK1α on phosphorylation of NSP5 in sites different from Serine 67

The phosphorylation of NSP5 is a hierarchical process where the first phosphorylation in ser-67 is followed by a series of other phosphorylations, probably in domain 4 (73), that give rise to the hyperphosphorylated forms of the protein. As previously mentioned, the phosphorylation on ser-67 can be mimicked by the substitution of the amino acid with an aspartic acid (NSP5/S67D). This mutant, indeed, when transfected alone in cells gets phosphorylated giving rise to slower migrating forms (figure 7). *In vitro* experiments had suggested that CK1α activity was responsible, at least in part, also for these steps of NSP5 phosphorylation. In order to evaluate this point we transfected the mutant NSP5/S67D together with si/irr or si/CK1α and evaluated the effect of the lack of CK1α on the phosphorylation of the protein. As expected (figure 23) on a PAGE mobility shift assay NSP5/S67D causes the appearance of slower migrating forms compared to the wt NSP5 (compare lane 1 with lane 2). However lack of CK1α does not have any influence on the formation of these slower migrating forms (compare lane 2 with lane 3). This suggests that kinases other than CK1α are involved in the further phosphorylation of NSP5.

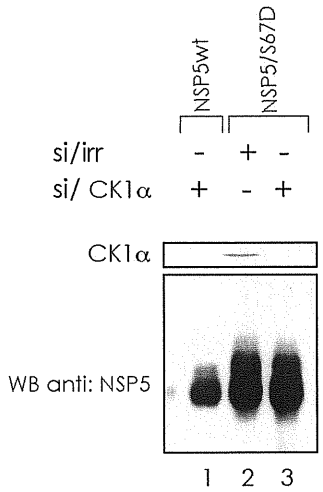


Figure 23: Expression of NSP5 wild type or NSP5/S67D in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect NSP5 and CK1α as indicated.

Effect of si/CK1α on the co-expression of NSP5 with NSP2

As mentioned in the introduction, NSP2 is able of activating NSP5 hyperphosphorylation *in vivo* when the two proteins are co-expressed in cells in absence of other viral proteins (figure 4) (5). In the model proposed NSP2 octamers interact with the N-terminal region of NSP5 and this favours the phosphorylation of NSP5 on serine 67, triggering the first step in the process of NSP5 hyperphosphorylation. In absence of the kinase responsible of the first step of phosphorylation, the interaction of NSP2 with NSP5 should not lead to the formation of the slower migrating bands seen in figure 4. In order to evaluate this point, cells were siRNA transfected and, 36 hrs after co-transfected with NSP5 and NSP2 under the control of T_7 promoter in presence of vaccinia virus. The virus causes the efficient expression of genes under the control of T_7 promoter in the cytoplasm. As it can be observed in figure 24, when NSP2 and NSP5 are co-expressed there is, as expected, an increase on the phosphorylation of NSP5 (red bracket). However, in presence of si/CK1α it can be observed a decrease in this phosphorylation demonstrating a role of CK1α in the hyperphosphorylation of NSP5 mediated by NSP2. This is an interesting result because, apart from confirming the results found with NSP5 mutants, it was obtained with the use of the wild type NSP5 and NSP2, strengthening the hypothesis of a role of CK1α in the phosphorylation of NSP5 also in the context of virus infection.

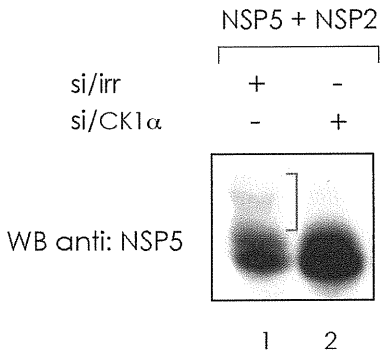


Figure 24: Coexpression of NSP5 and NSP2 in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect NSP5 with a specific antibody as indicated. Red bracket indicates the hyperphosphorylated form of NSP5.

Role of si/CK1α on NSP5 derived from virus infected cells

After having assessed the importance of CK1α on the phosphorylation of NSP5 in co-expression experiments, we decided to evaluate if the absence of CK1α had an effect also on NSP5 produced by virus infection. In order to do this, cells were transfected with the different si/RNAs and 48 h later, infected with rotavirus strains SA11 or OSU for 5 to 16 hrs, depending on the type of experiment performed. In a first type of experiment cells were siRNA-transfected and SA11-infected for 5 hrs and the status of NSP5 phosphorylation was evaluated through SDS-PAGE. In figure 25 it can be observed that whereas in the control or in cells treated with a non relevant siRNA (lanes 1 and 2) the most abundant forms of NSP5 are represented by the 28kDa one (empty triangle) and by the higher molecular weight forms, in the case of cells treated with si/CK1α, there is a strong decrease in the 28kDa and higher molecular weight forms and a consequent increase in the 26kDa form (black triangle). This demonstrates that also *in vivo* CK1α is responsible of phosphorylation of NSP5. It can be observed, however, that a certain quantity of 28kDa and hyperphosphorylated forms are still present. It remains to be elucidated if this is caused by NSP5 phosphorylation by kinases different from CK1α or is the result of NSP5 production by those cells that have not been transfected with the siRNA.

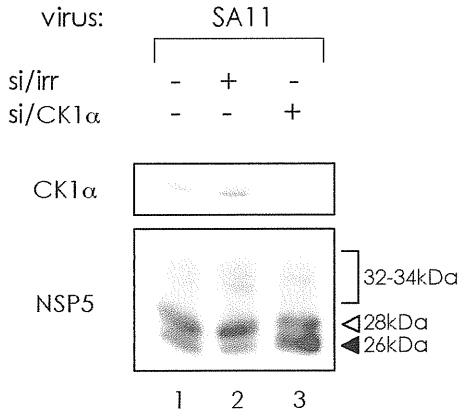


Figure 25: MA104 cells infected with SA11 strain in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect NSP5 and CK1α as indicated. Filled triangle indicates the 26kDa form of NSP5, empty one the 28kDa form and the bracket show the higher molecular weight forms.

In order to evaluate if the block of phosphorylation of NSP5 had an effect in the protein production by de novo DLPs and in the stability of NSP5, cells transfected with siRNAs and SA11-infected were treated with λ phosphatase. Previous results had demonstrated that in presence of λ phosphatase, all slower migrating bands of NSP5 can be eliminated with the formation of an unique band that migrates as 26kDa (figure 3) (6). As shown in figure 26 when the three extracts shown in figure 26A were treated with λ phosphatase (figure 26B) it

could be observed, as expected, the disappearance of the higher molecular weight bands and the formation of an unique band that migrates as 26kDa. It can be noted that the intensity of the bands of samples treated with λ phosphatase is comparable showing that lack of NSP5 phosphorylation does not affects NSP5 stability and protein production. It can also be observed that the process of dephosphorylation occurs to a slow rate also in absence of λ phosphatase probably for the presence of cellular phosphatases. Indeed samples incubated for two hours at 30°C in absence of λ phosphatase are less phosphorylated than the corresponding samples not incubated (compare samples 1, 2 or 3 of figure 26A with the corresponding samples of figure 26B).

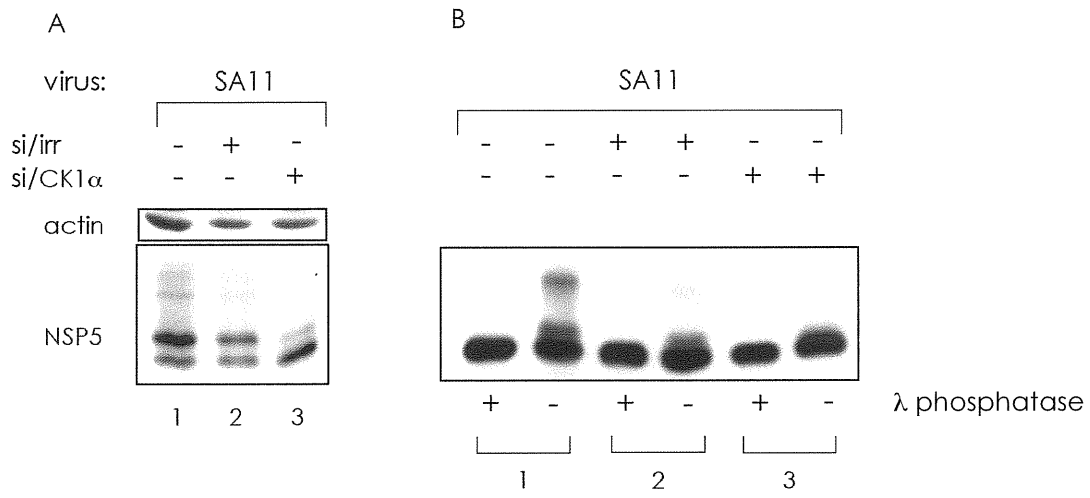


Figure 26: MA104 cells infected with SA11 strain in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. **(A)**Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect NSP5 and actin as indicated **(B)** the same samples were treated with λ phosphatase for 2 hours at 30°C and load on a SDS-PAGE and visualised by Western blot to detect NSP5.

Effect of siCK1α on the production of the other viral proteins

In the first part of this thesis we have shown that NSP5 is necessary for the production of viral proteins. Indeed in its absence, the production of the representative viral proteins (VP7, NSP2 and VP1) was inhibited. In order to verify if NSP5 phosphorylation could have a role in this process, we evaluated the production of viral proteins in presence of si/CK1α. Figure 27 shows the result of labelling total viral proteins with [^{35}S] methionine. As described in the introduction, upon viral infection, there is a shut off of the cellular protein synthesis and the translational machinery of the cell is diverted to the production of viral proteins. As it can be noticed in figure 27 if cells are infected and [^{35}S] methionine labelled, all the viral proteins

can be visualised in an SDS-PAGE (apart form NSP6) and proteins produced in not infected cells are not any longer visible (compare lane 1 with lane 4). It can be noticed that, even if the quantity of total protein produced is lower between mock transfected and siRNA transfected cells (probably a toxic effect due to the transfection procedure) there is no difference in the quantity of protein produced in presence of CK1α compared to the cells treated with an irrelevant si/RNA.

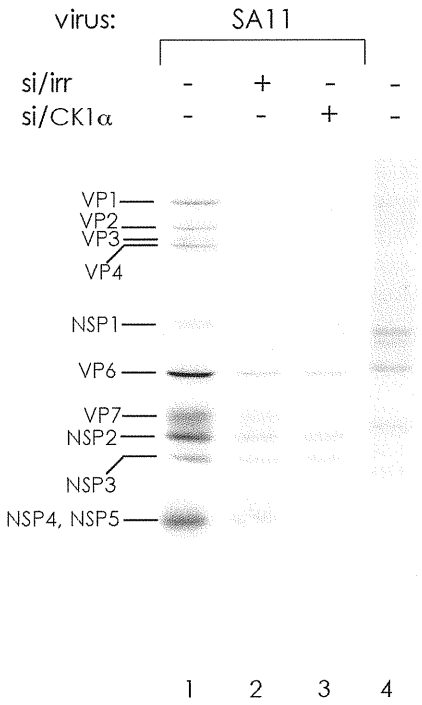


Figure 27: MA104 cells infected with SA11 strain in presence (+) of si/CK1α or an irrelevant si/RNA as indicated or not infected (lane 4). After 5 hours infection cells were labelled with [^{35}S] methionine and cellular extracts were loaded on a 12% SDS-PAGE gel to separate the proteins and visualised by autoradiography. Band attribution is shown on the left of the figure.

A confirmation of this kind of result has been obtained through western blot experiments. As shown in figure 28 the treatment of cells with si/CK1α (lane 1) does not affect the production of the non structural protein NSP2 in infected cells, confirming that in absence of NSP5 phosphorylation the viral protein production is not affected.

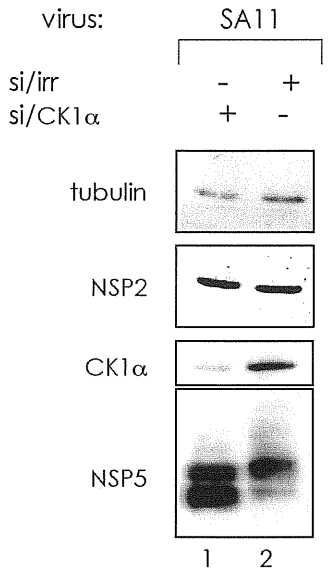


Figure 28: MA104 cells infected with SA11 strain in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 12% SDS-PAGE gel and visualised by Western blot to detect NSP5, tubulin, CK1α and NSP2 as indicated.

Interaction of NSP5 with other viral proteins

As described in the introduction, when infected cells are chemically crosslinked *in vivo* with DSP followed by immunoprecipitation with an antibody against NSP5, the two viral proteins VP1 and NSP2 co-immunoprecipitate together with NSP5. In addition, the two abundant viral proteins VP2 and VP6 were occasionally present in small amounts even if their presence was dependent on the stringency of washing performed (5). In order to evaluate if the phosphorylation of NSP5 had an effect on the interaction with these viral proteins, cells transfected with the siRNAs and infected with SA11 in the presence of [^{35}S] methionine, were crosslinked and immunoprecipitated with NSP5 antibody and resolved on a SDS-PAGE to visualise the presence of the cross-linked proteins. As it can be observed in figure 29, as expected, in mock infected cells, the antibody against NSP5 did not immunoprecipitate any protein showing its strong specificity for NSP5 (lane 4). It can be observed that NSP5 is less phosphorylated in si/CK1α treated cells (compare lane 3 with lane 2) confirming the data obtained through western blot. However we could not observe any difference in the interaction of NSP5 with either VP1 or NSP2, suggesting that the status of phosphorylation of NSP5 has not a role in its capability to interact with the viroplasmic resident proteins VP1 and NSP2.

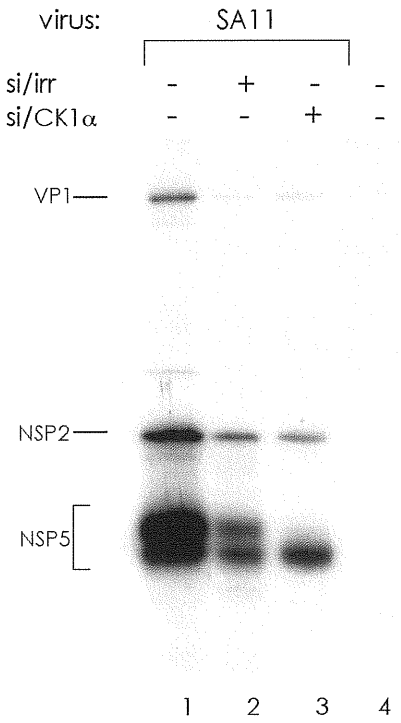


Figure 29: MA104 cells infected with SA11 strain in presence (+) of si/CK1α or an irrelevant si/RNA as indicated or not infected (lane 4). After 5 hours infection cells were labelled with [^{35}S] methionine and crosslinked with DSP. Cellular extracts were immunoprecipitated with an antibody against NSP5 and loaded on a 12% SDS-PAGE gel to separate the proteins. Protein presence was visualised by autoradiography. Band attribution is shown on the left of the figure.

Assessment of viroplasm formation in si/CK1α treated cells

As previously described, NSP5 localises in cytoplasmic viroplasms where viral replication and packaging occurs. In the previous part of our results we have demonstrated that in absence of NSP5 the production of viroplasms is completely impaired showing that NSP5 is necessary for the formation of viroplasms. In addition we have decided to evaluate the role of NSP5 phosphorylation on the viroplasms formation. Cells were transfected with either siRNA and, after 36-48 hrs infected for 5 hrs (figure 30A) or 16 hrs (figure 30B) with SA11. After infection cells were fixed and presence of viroplasms was visualised by immunofluorescence. Since also NSP2 localises in viroplasms together with NSP5, we analysed the formation of viroplasms through the use of an antibody against NSP2. In order to evaluate if in absence of CK1α there was an effect in the production of viroplasms, the number of infected cells was counted. As shown by histograms of representative experiments in figure 30, it could not be observed any relevant difference in the viroplasms formation at 5 and 16 hrs after infection suggesting that the formation of viroplasms was not influenced by the absence of CK1α.

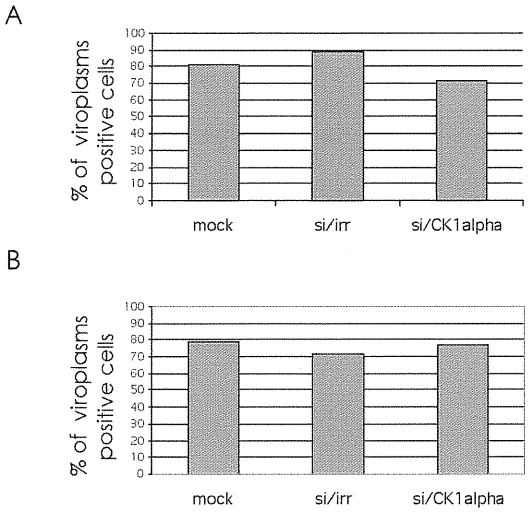


Figure 30: Histograms representing the percentage of infected cells in cells in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. **(A)** Cells infected with SA11 strain for 5 hours. **(B)** Cells infected with SA11 strain for 16 hours. Viroplasm positive cells were visualised in immunofluorescence through the use of an antibody against NSP2.

It is of interest to note that also after 16 hours of infection there was no difference in the pattern of phosphorylation of NSP5 in siCK1α-treated virus-infected cells compared with cells infected for 5 hrs as can be seen in figure 31 for virus strains SA11 (lanes 1-3) and OSU (lanes 4-6).

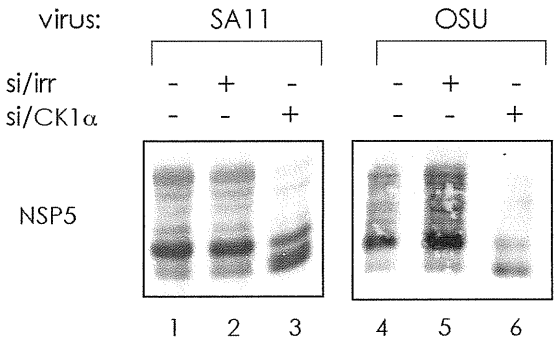


Figure 31: MA104 cells infected with SA11 or OSU strain for 16 hours in presence (+) of si/CK1α or an irrelevant si/RNA as indicated. Samples were loaded in a 15% SDS-PAGE gel and visualised by Western blot to detect NSP5 as indicated.

Even if the number of infected cells was comparable in control and si/CK1α treated cells at 5 hrs a more diffused fluorescence could be seen in cells transfected with si/CK1α. In order to evaluate if there was an effect in the formation of viroplasms in si/CK1α transfected cells we made a time course. For this reason cells were transfected for 48 hrs with siRNAs and infected for 5, 10 or 15 hrs with either SA11 or OSU virus strains.

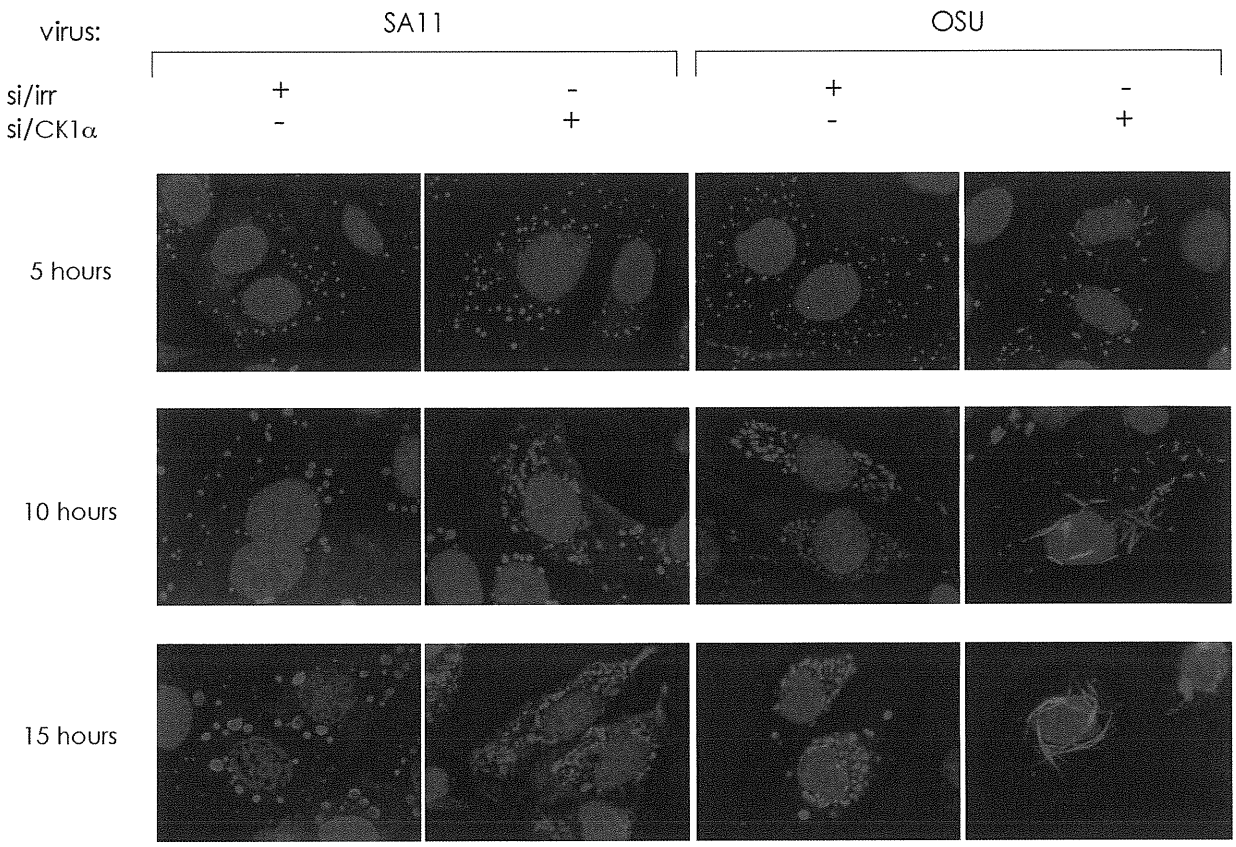


Figure 32: Time course of viroplasms formation in siRNA-treated cells. Immunofluorescence of MA104 cells transfected with si/CK1α or si/irr and infected with the indicated virus strain. Viroplasm were visualised with an anti-NSP2 antibody (red). Nuclei stained with Hoechst dye (blue).

In figure 32 it is possible to notice that the morphogenesis of viroplasms was strikingly different in the cells treated with si/CK1α compared with the normal morphogenesis of viroplasms formation. It has been demonstrated that upon infection, viroplasms are formed as soon as 2 hrs post infection and that from 6 hrs post infection, the total number of viroplasms per cell decreases with a concomitant increase in their size (72). We could observe the same pattern of morphogenesis in cells treated with si/irr and SA11 infected. As it can be observed in the first column of figure 32, as expected, we could notice that from 5 hrs to 15 hrs the number of viroplasms was decreasing whereas their size was increasing. The same pattern, even if less evident, could be observed in the case of cells infected with OSU strain (column 3). On the contrary the morphogenesis of viroplasms in cells treated with si/CK1α was quite different. If at short times after infection there could not be noticed practically no different in the shape of viroplasms, already at 10 hrs post infection the situation was different. As it can be noticed in the second column of figure 32, the viroplasms seemed not to be able to fuse to

form the regular spherical forms, instead, they were forming much more unstructured forms. Interestingly in many cells it could be observed conformations that resembled small viroplasms aggregating (figure 33A). Interestingly the morphogenesis of si/CK1α transfected/OSU infected cells was different from the one of SA11 cells. In this case indeed, viroplasms were assuming a more spiked form and the dimension of spikes was increasing in time (figure 32 first column). More detailed images of the structures formed are shown in figure 33B. However no clear cut difference could be noticed in the two strains since also in SA11 infected cells could be noticed, even if more rarely, spikes very similar to those found in OSU infected cells and, on converse, shapes resembling those found in SA11 infected cells could be found in the OSU infected ones.

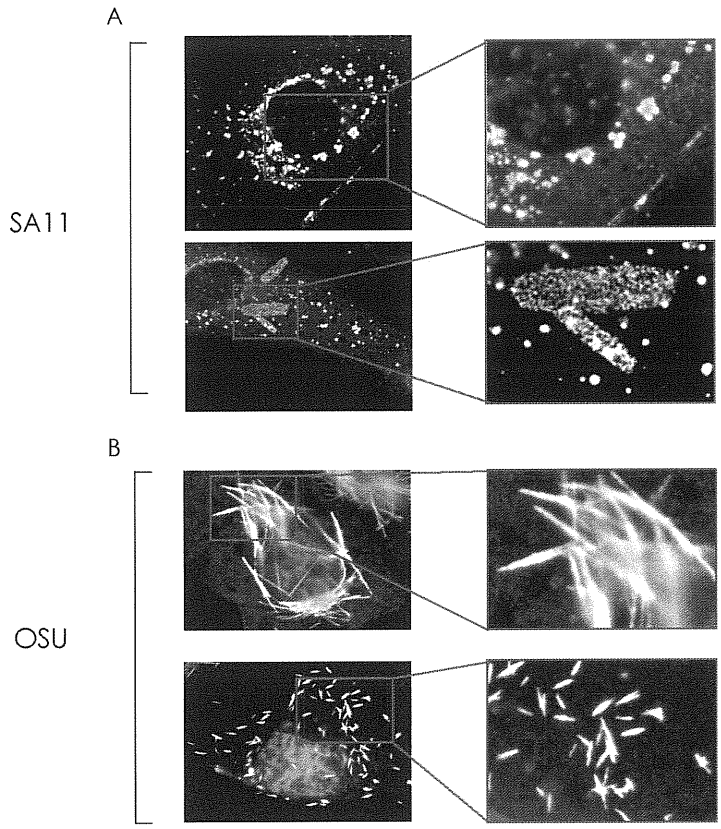


Figure 33: Viroplasms shape in si/CK1α treated cells. Immunofluorescence of MA104 cells transfected with si/CK1α and infected with the indicated virus strain. Viroplasm were visualised with an anti-NSP2 antibody.

In order to evaluate if, as a consequence of this altered structure of viroplasms, the colocalisation of NSP5 and NSP2 was different, cells siRNA transfected and SA11 infected were analysed, through immunofluorescence and confocal imaging, using simultaneously two antibodies against NSP5 and NSP2. As it is shown in figure 34, no difference could be noticed since the two proteins colocalised both in cells treated with an irrelevant siRNA and

the siRNA against CK1α. This is not a surprising result since in co-immunoprecipitation experiments, we could not notice any difference in the interaction between the two proteins.

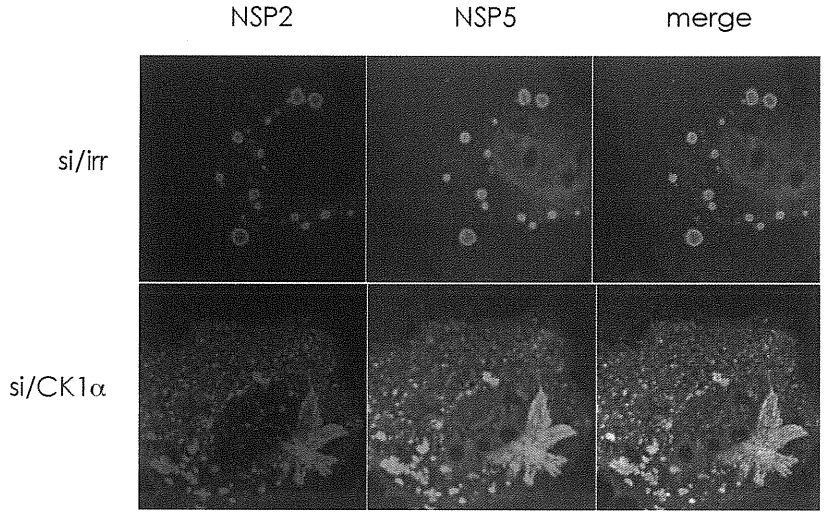


Figure 34: Confocal imaging of MA104 cells transfected with si/CK1α or si/irr and infected with SA11. Viroplasm were visualised with an anti-NSP2 antibody (red) and an anti NSP5 antibody (green). It is possible to visualise the colocalisation of the two proteins in viroplasms.

DISCUSSION

In many, but not all, group A rotaviruses, genomic segment 11 codes for two proteins, NSP5 and NSP6. NSP5 is an O-glycosylated phosphoprotein with a molecular mass of 26kDa that undergoes, upon viral infection, extensive phosphorylation with the appearance on SDS-PAGE, of slower migrating forms with an apparent molecular weight from 28 to 34kDa. Many biochemical studies have characterised the properties of NSP5 and it has been demonstrated that the process of NSP5 phosphorylation is quite complex involving the interaction of NSP5 with another non structural protein NSP2 and the action of cellular kinases in a process that is auto regulated by NSP5 itself (71, 73). NSP5 localises in viroplasms that are the site of rotavirus replication and packaging and in these structures NSP5 interacts with NSP2, VP1 and VP2. In addition NSP5, unique among the viral non structural proteins, interacts with both ssRNA and dsRNA in a sequence independent fashion. However, despite of the extensive characterisation of the biochemical properties of NSP5, its role was still unknown.

In order to unravel the role of NSP5 in the viral replicative cycle we decided to use the RNA interference approach. As described in the introduction this is a quite convenient method for the study of virus characteristics. The fact that the introduced siRNAs do not target a cellular protein render them particularly suitable for studies of viral protein functions since the effects obtained can be directly ascribed to the absence of the viral protein and not to some side effect due to the absence of a cellular protein. This technique is so powerful that is leading to a revolution in the field of virology as demonstrated by the fact that a huge number of studies have in the last few years used it to study viral proteins function. This is particularly interesting for rotavirus where the lack of a reverse genetic system has made the study of its viral protein functions particularly difficult.

To target the degradation of genomic segment 11 mRNA we designed two siRNAs specific for virus strains SA11 and OSU. A stretch of 19 nucleotides with a single nucleotide difference between the two strains was targeted with a siRNA specific for each of them. The nucleotide mismatch between the two sequences targeted is in position 93 of the gs11 mRNA corresponding to position 9 of the siRNAs. Both siRNAs showed to be extremely specific blocking the expression of NSP5 only from the virus encoding the fully homologous mRNA.

This strand specificity of the two siRNAs is in agreement with literature data showing that a single mismatch, particularly in position 9, can have a very strong effect on the activity and consequently specificity of the siRNAs (31, 89). The strand specificity provided an important internal control for the siRNAs effect, since it has been shown that some siRNAs are able of inducing the interferon response leading to a generalised shut off of the cell cycle (222). In our case the fact that the siRNAs showed a strong strand specificity allowed us to rule out a generalised effect in the siRNA treated cells since they were able to allow replication of the non-homologous viral strain to a level comparable to the one of in untransfected cells.

Inhibition of gs11 had a strong effect in the replicative cycle of the virus. We could show that in siRNA-treated virus-infected cells there was an almost complete inhibition of viroplasms formation. This result is in agreement with those we obtained through the use of intracellular antibodies against NSP5. Also in this case the presence of ICAbs specific for NSP5 caused a block of the formation of viroplasms in infected cells. Taken together these results show the essential role of NSP5 in the viroplasm formation. In addition we were able to complement for viroplasms formation in si/SA11-transfected SA11-infected cells through the use of a stable cell line expressing OSU NSP5. This kind of experiments has important consequences since directly ascribe to NSP5 the effect found in the inhibition viroplasms formation. As mentioned in the introduction (§ 1.4.6.) and shown in the results (figure 11), OSU genomic segment 11 codes for a truncated form of NSP6 that reduces the ORF to only 51 amino acids respect to the 93 residues of the SA11 strain. Although the siRNAs used inhibit the synthesis of both NSP5 and NSP6, the fact that we were able to complement for viroplasm formation with a construct derived from OSU genomic segment 11 shows that NSP6 is not a necessary protein for the viroplasm formation. This kind of results is furthermore supported by the fact that ICAbs against NSP5 that, consequently, do not target NSP6, show the same viroplasm negative phenotype observed with RNA interference against genome segment 11. In addition a recent paper, published just after the one that describes the results obtained in the first part of this thesis, has shown that the inhibition of NSP5 by RNA interference in the virus strain Alabama, that does not express NSP6, had the same effect on replication and protein synthesis to the one of another strain, RRV, that contained a full length NSP6 (148). Taken together these data demonstrate that NSP6 is not a necessary protein for the replication of rotavirus. The role, if any, of this protein has still to be elucidated.

Lack of NSP5 caused also a very strong inhibition of dsRNA production and a decrease of virus titers of more than 10 fold due, probably, to the lack of viroplasms formation.

However virus production was not completely abolished probably because not all cells were transfected as demonstrated by the use of a fluoresceinated siRNA. The result obtained resembles the phenotype of temperature sensitive mutants affecting the structural proteins involved in virus replication VP1 (*tsC*), VP3 (*tsB*) (41) as well as the non structural protein NSP2 (*tsE*) that were characterised by a lack of viroplasms formation and impaired viral ssRNA and dsRNA production (41, 205, 232). In addition a practically identical phenotype was observed in cells that were depleted of NSP2 by RNA interference that showed also a decrease in virus titers that was of the same order of magnitude of the one we observed (221). These results confirm the importance of the interaction between NSP5 and NSP2 in the virus replicative cycle.

Another interesting information that can be obtained by RNA interference of rotaviral NSP5 regards the viral secondary transcription. We could observe that in cells depleted of NSP5 there was a concomitant significative decrease in the production of all the other viral proteins. This suggests that most of the viral proteins found in virus infected cells are, rather than the production of the internalised infecting virions, the consequence of translation of secondary transcripts produced by de novo synthesised particles that have not yet completed the maturation steps. Similar results have been obtained through RNA interference against NSP2 (221). In addition this observation is in agreement with results on the time course of mRNA production in virus infected cells that have shown how after about three hours post infection there is a strong increase in mRNA production due to the assembly of transcriptionally active DLPs for secondary transcription (224). This kind of interpretation on viral transcription is also consistent with the active transcription of viral genes occurring in DLPs derived from purified mature infectious virions (127, 137, 180, 234).

An interesting result obtained in our study was the possibility to complement, even though partially, with an exogenous gene for the lack of NSP5 both in the formation of viroplasms and in viral genome replication. This is, to our knowledge, the first and only description of a complementation experiment in rotavirus. Since the lack of a reverse genetic system for rotavirus has not allowed up to now this kind of experiments, the RNA interference approach, in combination with complementation assays as the one utilised in our work, opens new opportunities to understand the function of rotaviral proteins through the use of protein mutants.

Another aim of our work was the identification of the kinase responsible of the phosphorylation of NSP5 and the role of this phosphorylation in virus infection.

As described in the introduction, previous results obtained in this laboratory, had allowed to uncouple activation and substrate function of NSP5 through the use of deletion mutants. The mutant lacking region 2 showed to be a good substrate since it did not get phosphorylated unless in the presence of an activator molecule such as Δ3. The use of these mutants allowed having an insight in the process of NSP5 phosphorylation determining that NSP5 auto regulates its own phosphorylation in a multistep process in which NSP2 has a determinant role (71, 73). Another result obtained was the determination of phosphorylation on serine 67 to be the necessary initiation step in the process of NSP5 phosphorylation and in the identification of CK1α as the kinase phosphorylating NSP5 on serine 67. In addition *in vitro* experiments had shown that CK1α was also responsible of the phosphorylations causing the formation of higher molecular weight forms (71).

In order to confirm the role of CK1α in the phosphorylation of NSP5, we decided to use the RNA interference approach in order to eliminate CK1α and evaluate the effect on NSP5. Based on previous reports that achieved a strong inhibition of CK1α expression through RNAi we decided to target a region of CK1α that goes from nucleotide 151 to 173 of the ORF of CK1α (107, 143).

Also in this case the use of RNA interference demonstrated to be a very efficient method for the elimination of the target protein since, with a single transfection of the siRNA we were able of strongly blocking the cellular production of CK1α. The use of this siRNA in co-expression experiments allowed us to confirm that CK1α is the kinase responsible of NSP5 phosphorylation in serine 67 *in vivo*. Indeed, co-expression of SV5-Δ2 together with Δ3 in absence of CK1α caused a strong decrease in the phosphorylation of SV5-Δ2 most likely as a consequence of the lack of phosphorylation of Δ3 on serine 67. The inhibition observed was, however, not total suggesting that some other kinase was also involved in the phosphorylation. In agreement with this result is the fact that cotransfection of NSP2 together with NSP5 failed to cause hyperphosphorylation of NSP5 when a siRNA against CK1α was used. These results strongly support the determinant role of CK1α on NSP5 phosphorylation and confirm previous results obtained during *in vitro* experiments (71).

The results obtained with the use of the NSP5 mutant NSP5/S67D, instead diverge with what was previously found. Indeed in our hands there was no effect on the

hyperphosphorylation status of NSP5/S67D when CK1α expression was abolished. Since the hyperphosphorylation of NSP5 is thought to be caused not by the addition of a phosphate on serine 67 but by the subsequent steps of phosphorylation, these result suggest that kinases other that CK1α are responsible for these further steps of phosphorylation. The discrepancy between our data and the previous ones can be ascribed to the method used. Indeed the previous results were obtained through an *in vitro* phosphorylation assay where the protein was phosphorylated through the addition of recombinant zebrafish CK1α whereas our results were obtained in the more physiological environment of transfected cells. However further studies in which NSP5 mutants will be exposed to cellular extracts in presence or absence of si/CK1α during *in vitro* kinase assays should help to clarify this point. If these data are confirmed it will remain to identify the kinase responsible of the subsequent phosphorylations of NSP5.

In addition to this kind of experiments, we also decided to assess the role of NSP5 phosphorylation in the replicative cycle of the virus. In order to do this, we transfected MA104 with si/CK1α and infected cells with either SA11 or OSU rotavirus strains. We could notice that lack of CK1α had a strong effect on the phosphorylation of NSP5 both at short and long times of infection. It was possible to notice an inversion between the appearance of the 26 and 28kDa since in cells mock transfected or transfected with an irrelevant siRNA the most represented band corresponded to the one of 28 kDa whereas in cells treated with si/CK1α the most represented band was the 26kDa one, it could also be noticed a decrease in the higher molecular weight forms. If this kind of pattern was already clear at 5 hrs after infection it was as well evident when cells were infected for a longer time (16 hrs); also in this case it could be noticed that a part from the increase of the 26kDa band there was also a marked decrease in the higher molecular weight ones (34kDa) (figure 31). These results suggest that the slower migrating forms of NSP5 are the precursor of higher forms and that, in absence of the priming phosphorylation of NSP5 by CK1α on serine 67, the formation of the slower migrating forms is inhibited. This interpretation is in agreement with literature data showing that the 26kDa form of NSP5 is a precursor for the apparent higher molecular weight forms of the protein (6).

The fact of having obtained a significative block of NSP5 phosphorylation during in vivo infection experiments with a rather simple and direct method prompted us to perform a series of experiments to try to understand the role of NSP5 in the virus replicative cycle.

An interesting information was regarding the viroplasm formation in absence of NSP5 phosphorylation. This is not the first study that tried to investigate this topic since previous studies had tried to link the phosphorylation of NSP5 with its localisation in viroplasms with contrasting results. Blackhall *et al.* had shown that in early times after virus infection (4 hours) the most fast migrating bands of NSP5 (26 and 28 kDa) were the most represented whereas the slower migrating bands (30-35 kDa) were not present and appeared later in virus infection (from 6 hours); since the authors could notice that even at early times after infection (2 hours) NSP5 was localising in viroplasms they deduced that NSP5 phosphorylation was not necessary for viroplasm localisation. However previous results of this laboratory had shown that, dissimilarly from what found by Blackhall and colleagues, already at 4 hours after infection NSP5 is already phosphorylated showing the same phosphorylation bands that are evidenced at longer infection times (6). Other results of this laboratory had further shown that localisation in viroplasms was not dependent on phosphorylation since also NSP5 deletion mutants that are not phosphorylated localised in viroplasms in infected cells (72, 73). This kind of study, however did not allow the evaluation of the localisation of the protein in absence of the wild type form of NSP5 because the mutant was transfected in cells that were subsequently infected with the virus. Since it is known that NSP5 forms dimers (195, 235) the interaction between NSP5 wt and the deletion mutants could have been responsible of their localisation in viroplasms. On the contrary, another study had suggested that NSP5 phosphorylation and localisation in viroplasms were linked events (195).

The use of RNA interference and its high efficiency in blocking NSP5 phosphorylation allowed us to analyse the viroplasms formation directly of in virus infected cells expressing a wild type form of the protein. We could notice that phosphorylation had no effect on the first phases of viroplasms formation since in the first hours the shape of viroplasms was similar to the one of cells transfected with a non relevant siRNA. However in the cytoplasm of cells lacking CK1α we could notice a more diffuse punctuated pattern. At longer times post infection, the effect was even more striking. In SA11 infected cells indeed, the shape of viroplasms was completely subverted and instead of being very ordered spherical structures localised mainly around the nucleus, viroplasms showed a quite irregular form and very often it could be noticed the presence of structures that resembled an unsuccessful attempt of viroplasms to fuse. This suggests that the role of NSP5 phosphorylation is not the one of allowing the formation of viroplasms but instead it seems to have a role in allowing the correct viroplasms morphogenesis and fusion. Interestingly the viroplasms shape in OSU

infected cells was markedly different from the one observed in SA11 cells. Actually the morphogenesis of OSU viroplasms was different from the one observed in SA11 cells also in not transfected cells, even if cells were infected with comparable quantity of virus. Indeed in this case at longer times it was possible to notice a major number of viroplasms of smaller size and their shape was not so well defined as in the case of SA11 infected cells. When RNA interference was used, in absence of CK1α, viroplasms were spiked instead of spherical and the size of these viroplasms was increasing in time. Even at 5 hours a tendency of some viroplasms to have a more spiked form could be observed. This difference in viroplasms shape is possibly due to differences in the NSP5 sequence among the two virus strains and experiments to assess this possibility are currently under investigation.

It is not possible to directly ascribe the effect we have noticed in viroplasm formation in absence of CK1α on NSP5 since the lack of phosphorylation of some other cellular or viral protein could be responsible of the effect observed. However, the fact that NSP5 localises in viroplasms and that, as we have shown in the first part of this thesis, is necessary for the formation of viroplasms together with the direct role of CK1α on NSP5 phosphorylation, strongly address the lack of phosphorylation of this protein as the responsible for the phenotype observed. In addition the fact that the morphogenesis of viroplasms is different in the two virus strains used suggests that the effect on a viral protein is responsible of the impaired morphogenesis: if the target would have been a cellular protein, we would have observed the same phenotype with the two virus strains.

We could not notice any significant difference in the localisation of viroplasms in siRNA treated and not treated cells that were, in both cases, in a perinuclear localisation, suggesting that viroplasms correct morphogenesis and localisation are two separated processes and that NSP5 phosphorylation seems not to have a role in the viroplasms movement. In addition we could not notice any difference in the colocalisation of NSP5 and NSP2 in viroplasms. This was not surprising since cross linking experiments of siRNA treated-virus infected cells had shown no difference in the interaction between the two proteins.

Since secondary transcription, responsible of the production of the majority of viral proteins as we and other have shown (this thesis and (148, 221)), occurs in cytoplasmic viroplasms, we wanted to investigate if their altered shape due to treatment with si/CK1α could have an effect on viral protein production. In order to assess this point we performed a series of experiments to identify viral proteins such as labelling of total viral proteins with [^{35}S] methionine or use of specific antibodies in Western blot analysis. All these results

showed that there was no difference in the viral protein production between cells treated with an irrelevant siRNA or those treated with a siRNA against CK1α. This is an indication that even if viroplasms are more in number but smaller and not present a wild type shape this has no effect on the production of viral proteins. This is an interesting result since it suggests that NSP5 phosphorylation does not have a role in the exit of viral mRNAs formed by newly assembled particles in viroplasms nor in controlling translation of viral mRNAs.

In co-immunoprecipitation experiments it has been shown that NSP5 interacts both with NSP2 and with the viral polymerase VP1. We could not observe any difference in the protein interaction upon transfection of si/CK1α and infection suggesting that the phosphorylation pattern of NSP5 has no effect on the capability of interaction with viral proteins. This result is in agreement with previous studies that had shown as the interaction between VP2 and NSP5 did not depend on the status of NSP5 phosphorylation (18) as well as in studies with deletion mutants of NSP5, where, similarly, the interaction with NSP2 was not determined by the status of phosphorylation of the mutant (73).

Interestingly, very recently, it was published a paper about the protein NS2 of bluetongue virus (another member of the *Reoviridae* family) (166). NS2 is the major component of viral inclusion bodies (VIBs) that correspond to rotavirus viroplasms. Recombinant NS2, when expressed in absence of other viral proteins, is able of forming intracellular aggregates that resemble VIBs. NS2 is phosphorylated but does not get hyperphosphorylated and shares some characteristics with rotavirus NSP2 such as NTPase activity and affinity for ssRNA that have suggested the two proteins share similar functions. In their study, Modrof and colleagues identified the sites of phosphorylation of NS2 and showed the protein to be phosphorylated by casein kinase 2 (CK2). In addition, through the use of a NS2 mutant that does not gets phosphorylated the authors showed that NS2 could interact with viral inner core proteins regardless of its status of phosphorylation. However this mutant was not able of forming VIB (166). Even if a direct correlation of NSP5 with NS2 is not possible, the two proteins share some common features: both proteins are phosphorylated by members of the casein kinase family and NSP5 in certain conditions is able to form structures resembling viroplasms when expressed in absence of other viral proteins as NS2 (167). The fact that lack of phosphorylation in both proteins does not affect viral protein production and interaction but impairs the viroplasm (or VIBs) assembly, suggests a common role for the two proteins.

Taken together the results presented demonstrate the essential role of NSP5 in the viral replicative cycle of rotavirus since its absence causes block of viral protein production, of viroplasms formation and of genome replication. In addition we were able to confirm through *in vivo* experiments, that CK1α is the kinase responsible of NSP5 phosphorylation on serine 67. However our data suggest that kinases other that CK1α are involved in the subsequent phosphorylations of NSP5. We could also assess the role of NSP5 phosphorylation in the replicative cycle of rotavirus. These results allowed us to demonstrate that NSP5 phosphorylation does not have an effect on viral protein production or in the interaction of NSP5 with other viroplasm resident proteins but is involved in the correct morphogenesis of viroplasms. This is, to our knowledge, the first indication of a role of NSP5 in the replication of rotavirus.

References

1. **Abdul-Ghani, M., P. Y. Gougeon, D. C. Prosser, L. F. Da-Silva, and J. K. Ngsee.** 2001. PRA isoforms are targeted to distinct membrane compartments. J Biol Chem **276:**6225-33.

2. **Adelman, Z. N., C. D. Blair, J. O. Carlson, B. J. Beaty, and K. E. Olson.** 2001. Sindbis virus-induced silencing of dengue viruses in mosquitoes. Insect Mol Biol **10:**265-73.

3. **Adelman, Z. N., I. Sanchez-Vargas, E. A. Travanty, J. O. Carlson, B. J. Beaty, C. D. Blair, and K. E. Olson.** 2002. RNA silencing of dengue virus type 2 replication in transformed C6/36 mosquito cells transcribing an inverted-repeat RNA derived from the virus genome. J Virol **76:**12925-33.

4. **Affranchino, J. L., and S. A. Gonzalez.** 1997. Deletion mapping of functional domains in the rotavirus capsid protein VP6. J Gen Virol **78 (Pt 8):**1949-55.

5. **Afrikanova, I., E. Fabbretti, M. C. Miozzo, and O. R. Burrone.** 1998. Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2. J Gen Virol **79 (Pt 11):**2679-86.

6. **Afrikanova, I., M. C. Miozzo, S. Giambiagi, and O. Burrone.** 1996. Phosphorylation generates different forms of rotavirus NSP5. J Gen Virol **77 (Pt 9):**2059-65.

7. **Aponte, C., D. Poncet, and J. Cohen.** 1996. Recovery and characterization of a replicase complex in rotavirus-infected cells by using a monoclonal antibody against NSP2. J Virol **70:**985-91.

8. **Araujo, I. T., M. S. Ferreira, A. M. Fialho, R. M. Assis, C. M. Cruz, M. Rocha, and J. P. Leite.** 2001. Rotavirus genotypes P[4]G9, P[6]G9, and P[8]G9 in hospitalized children with acute gastroenteritis in Rio de Janeiro, Brazil. J Clin Microbiol **39:**1999-2001.

9. **Arias, C. F., P. Romero, V. Alvarez, and S. Lopez.** 1996. Trypsin activation pathway of rotavirus infectivity. J Virol **70:**5832-9.

10. **Arista, S., E. Vizzi, M. C. Migliore, E. Di Rosa, and A. Cascio.** 2003. High incidence of G9P181 rotavirus infections in Italian children during the winter season 1999-2000. Eur J Epidemiol **18:**711-4.

11. **Au, K. S., W. K. Chan, J. W. Burns, and M. K. Estes.** 1989. Receptor activity of rotavirus nonstructural glycoprotein NS28. J Virol **63:**4553-62.

12. **Au, K. S., N. M. Mattion, and M. K. Estes.** 1993. A subviral particle binding domain on the rotavirus nonstructural glycoprotein NS28. Virology **194:**665-73.

13. **Au, W. C., P. A. Moore, W. Lowther, Y. T. Juang, and P. M. Pitha.** 1995. Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. Proc Natl Acad Sci U S A **92:**11657-61.

14. **Ball, J. M., P. Tian, C. Q. Zeng, A. P. Morris, and M. K. Estes.** 1996. Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. Science **272:**101-4.

15. **Barro, M., and J. T. Patton.** 2005. Rotavirus nonstructural protein 1 subverts innate immune response by inducing degradation of IFN regulatory factor 3. Proc Natl Acad Sci U S A **102:**4114-9.

16. **Bass, D. M., M. R. Baylor, C. Chen, E. M. Mackow, M. Bremont, and H. B. Greenberg.** 1992. Liposome-mediated transfection of intact viral particles reveals that plasma membrane penetration determines permissivity of tissue culture cells to rotavirus. J Clin Invest **90:**2313-20.

17. **Bennasser, Y., S. Y. Le, M. Benkirane, and K. T. Jeang.** 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity **22:**607-19.

18. **Berois, M., C. Sapin, I. Erk, D. Poncet, and J. Cohen.** 2003. Rotavirus nonstructural protein NSP5 interacts with major core protein VP2. J Virol **77:**1757-63.

19. **Bican, P., J. Cohen, A. Charpilienne, and R. Scherrer.** 1982. Purification and characterization of bovine rotavirus cores. J Virol **43:**1113-7.

20. **Bishop, R. F., G. P. Davidson, I. H. Holmes, and B. J. Ruck.** 1973. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet **2:**1281-3.

21. **Bitko, V., A. Musiyenko, O. Shulyayeva, and S. Barik.** 2005. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med **11:**50-5.

22. **Blackhall, J., A. Fuentes, K. Hansen, and G. Magnusson.** 1997. Serine protein kinase activity associated with rotavirus phosphoprotein NSP5. J Virol **71:**138-44.

23. **Blackhall, J., M. Munoz, A. Fuentes, and G. Magnusson.** 1998. Analysis of rotavirus nonstructural protein NSP5 phosphorylation. J Virol **72:**6398-405.

24. **Boden, D., O. Pusch, F. Lee, L. Tucker, and B. Ramratnam.** 2003. Human immunodeficiency virus type 1 escape from RNA interference. J Virol **77:**11531-5.

25. **Bok, K., G. Palacios, K. Sijvarger, D. Matson, and J. Gomez.** 2001. Emergence of G9 P[6] human rotaviruses in Argentina: phylogenetic relationships among G9 strains. J Clin Microbiol **39:**4020-5.

26. **Bon, F., C. Fromantin, S. Aho, P. Pothier, and E. Kohli.** 2000. G and P genotyping of rotavirus strains circulating in france over a three-year period: detection of G9 and P[6] strains at low frequencies. The AZAY Group. J Clin Microbiol **38:**1681-3.

27. **Boshuizen, J. A., J. W. Rossen, C. K. Sitaram, F. F. Kimenai, Y. Simons-Oosterhuis, C. Laffeber, H. A. Buller, and A. W. Einerhand.** 2004. Rotavirus enterotoxin NSP4 binds to the extracellular matrix proteins laminin-beta3 and fibronectin. J Virol **78:**10045-53.

28. **Bowman, G. D., I. M. Nodelman, O. Levy, S. L. Lin, P. Tian, T. J. Zamb, S. A. Udem, B. Venkataraghavan, and C. E. Schutt.** 2000. Crystal structure of the oligomerization domain of NSP4 from rotavirus reveals a core metal-binding site. J Mol Biol **304:**861-71.

29. **Boyle, J. F., and K. V. Holmes.** 1986. RNA-binding proteins of bovine rotavirus. J Virol **58:**561-8.

30. **Brottier, P., P. Nandi, M. Bremont, and J. Cohen.** 1992. Bovine rotavirus segment 5 protein expressed in the baculovirus system interacts with zinc and RNA. J Gen Virol **73 (Pt 8):**1931-8.

31. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550-3.

32. **Burns, J. W., M. Siadat-Pajouh, A. A. Krishnaney, and H. B. Greenberg.** 1996. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science **272:**104-7.

33. **Burzio, V., M. Antonelli, C. C. Allende, and J. E. Allende.** 2002. Biochemical and cellular characteristics of the four splice variants of protein kinase CK1alpha from zebrafish (Danio rerio). J Cell Biochem **86:**805-14.

34. **Carpio, R. V., F. D. Gonzalez-Nilo, H. Jayaram, E. Spencer, B. V. Prasad, J. T. Patton, and Z. F. Taraporewala.** 2004. Role of the histidine triad-like motif in nucleotide hydrolysis by the rotavirus RNA-packaging protein NSP2. J Biol Chem **279:**10624-33.

35. **Cartee, T. L., and G. W. Wertz.** 2001. Respiratory syncytial virus M2-1 protein requires phosphorylation for efficient function and binds viral RNA during infection. J Virol **75:**12188-97.

36. **Cegielska, A., and D. M. Virshup.** 1993. Control of simian virus 40 DNA replication by the HeLa cell nuclear kinase casein kinase I. Mol Cell Biol **13:**1202-11.

37. **Charpilienne, A., M. J. Abad, F. Michelangeli, F. Alvarado, M. Vasseur, J. Cohen, and M. C. Ruiz.** 1997. Solubilized and cleaved VP7, the outer glycoprotein of rotavirus, induces permeabilization of cell membrane vesicles. J Gen Virol **78 (Pt 6):**1367-71.

38. **Charpilienne, A., J. Lepault, F. Rey, and J. Cohen.** 2002. Identification of rotavirus VP6 residues located at the interface with VP2 that are essential for capsid assembly and transcriptase activity. J Virol **76:**7822-31.

39. **Chemello, M. E., O. C. Aristimuno, F. Michelangeli, and M. C. Ruiz.** 2002. Requirement for vacuolar H+ - ATPase activity and Ca2+ gradient during entry of rotavirus into MA104 cells. J Virol **76:**13083-7.

40. **Chen, D., M. Barros, E. Spencer, and J. T. Patton.** 2001. Features of the 3'-consensus sequence of rotavirus mRNAs critical to minus strand synthesis. Virology **282:**221-9.

41. **Chen, D., J. L. Gombold, and R. F. Ramig.** 1990. Intracellular RNA synthesis directed by temperature-sensitive mutants of simian rotavirus SA11. Virology **178:**143-51.

42. **Chen, D., C. L. Luongo, M. L. Nibert, and J. T. Patton.** 1999. Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase. Virology **265:**120-30.

43. **Chen, D., and J. T. Patton.** 1998. Rotavirus RNA replication requires a single-stranded 3' end for efficient minus-strand synthesis. J Virol **72:**7387-96.

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

45. **Chizhikov, V., and J. T. Patton.** 2000. A four-nucleotide translation enhancer in the 3'-terminal consensus sequence of the nonpolyadenylated mRNAs of rotavirus. Rna **6:**814-25.

46. **Ciarlet, M., and M. K. Estes.** 1999. Human and most animal rotavirus strains do not require the presence of sialic acid on the cell surface for efficient infectivity. J Gen Virol **80 (Pt 4):**943-8.

47. **Clarke, M. L., L. J. Lockett, and G. W. Both.** 1995. Membrane binding and endoplasmic reticulum retention sequences of rotavirus VP7 are distinct: role of carboxy-terminal and other residues in membrane binding. J Virol **69:**6473-8.

48. **Cohen, J., A. Charpilienne, S. Chilmonczyk, and M. K. Estes.** 1989. Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. Virology **171:**131-40.

49. **Cohen, J., J. Laporte, A. Charpilienne, and R. Scherrer.** 1979. Activation of rotavirus RNA polymerase by calcium chelation. Arch Virol **60:**177-86.

50. **Colbere-Garapin, F., B. Blondel, A. Saulnier, I. Pelletier, and K. Labadie.** 2005. Silencing viruses by RNA interference. Microbes Infect **7:**767-75.

51. **Coulson, B. S., S. L. Londrigan, and D. J. Lee.** 1997. Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells. Proc Natl Acad Sci U S A **94:**5389-94.

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

53. **Crawford, S. E., S. K. Mukherjee, M. K. Estes, J. A. Lawton, A. L. Shaw, R. F. Ramig, and B. V. Prasad.** 2001. Trypsin cleavage stabilizes the rotavirus VP4 spike. J Virol **75:**6052-61.

54. **Cuadras, M. A., C. F. Arias, and S. Lopez.** 1997. Rotaviruses induce an early membrane permeabilization of MA104 cells and do not require a low intracellular Ca2+ concentration to initiate their replication cycle. J Virol **71:**9065-74.

55. **Cuadras, M. A., and H. B. Greenberg.** 2003. Rotavirus infectious particles use lipid rafts during replication for transport to the cell surface in vitro and in vivo. Virology **313:**308-21.

56. **Cunliffe, N. A., W. Dove, J. E. Bunn, M. Ben Ramadam, J. W. Nyangao, R. L. Riveron, L. E. Cuevas, and C. A. Hart.** 2001. Expanding global distribution of rotavirus serotype G9: detection in Libya, Kenya, and Cuba. Emerg Infect Dis **7:**890-2.

57. **Das, A. T., T. R. Brummelkamp, E. M. Westerhout, M. Vink, M. Madiredjo, R. Bernards, and B. Berkhout.** 2004. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol **78:**2601-5.

58. **Dector, M. A., P. Romero, S. Lopez, and C. F. Arias.** 2002. Rotavirus gene silencing by small interfering RNAs. EMBO Rep **3:**1175-80.

59. **Delmas, O., A. M. Durand-Schneider, J. Cohen, O. Colard, and G. Trugnan.** 2004. Spike protein VP4 assembly with maturing rotavirus requires a postendoplasmic reticulum event in polarized caco-2 cells. J Virol **78:**10987-94.

60. **Delmas, O., A. Gardet, S. Chwetzoff, M. Breton, J. Cohen, O. Colard, C. Sapin, and G. Trugnan.** 2004. Different ways to reach the top of a cell. Analysis of rotavirus assembly and targeting in human intestinal cells reveals an original raft-dependent, Golgi-independent apical targeting pathway. Virology **327:**157-61.

61. **Denisova, E., W. Dowling, R. LaMonica, R. Shaw, S. Scarlata, F. Ruggeri, and E. R. Mackow.** 1999. Rotavirus capsid protein VP5* permeabilizes membranes. J Virol **73:**3147-53.

62. **Deo, R. C., C. M. Groft, K. R. Rajashankar, and S. K. Burley.** 2002. Recognition of the rotavirus mRNA 3' consensus by an asymmetric NSP3 homodimer. Cell **108:**71-81.

63. **Desselberger, U., and M. A. McCrae.** 1994. The rotavirus genome. Curr Top Microbiol Immunol **185:**31-66.

64. **Dormitzer, P. R., and H. B. Greenberg.** 1992. Calcium chelation induces a conformational change in recombinant herpes simplex virus-1-expressed rotavirus VP7. Virology **189:**828-32.

65. **Dormitzer, P. R., H. B. Greenberg, and S. C. Harrison.** 2001. Proteolysis of monomeric recombinant rotavirus VP4 yields an oligomeric VP5* core. J Virol **75:**7339-50.

66. **Dormitzer, P. R., H. B. Greenberg, and S. C. Harrison.** 2000. Purified recombinant rotavirus VP7 forms soluble, calcium-dependent trimers. Virology **277:**420-8.

67. **Dormitzer, P. R., E. B. Nason, B. V. Prasad, and S. C. Harrison.** 2004. Structural rearrangements in the membrane penetration protein of a non-enveloped virus. Nature **430:**1053-8.

68. **Dormitzer, P. R., Z. Y. Sun, G. Wagner, and S. C. Harrison.** 2002. The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. Embo J **21:**885-97.

69. **Dowling, W., E. Denisova, R. LaMonica, and E. R. Mackow.** 2000. Selective membrane permeabilization by the rotavirus VP5* protein is abrogated by mutations in an internal hydrophobic domain. J Virol **74:**6368-76.

70. **Dunn, S. J., T. L. Cross, and H. B. Greenberg.** 1994. Comparison of the rotavirus nonstructural protein NSP1 (NS53) from different species by sequence analysis and northern blot hybridization. Virology **203:**178-83.

71. **Eichwald, C., G. Jacob, B. Muszynski, J. E. Allende, and O. R. Burrone.** 2004. Uncoupling substrate and activation functions of rotavirus NSP5: phosphorylation of Ser-67 by casein kinase 1 is essential for hyperphosphorylation. Proc Natl Acad Sci U S A **101:**16304-9.

72. **Eichwald, C., J. F. Rodriguez, and O. R. Burrone.** 2004. Characterisation of rotavirus NSP2/NSP5 interaction and dynamics of viroplasms formation. J Gen Virol **85:**625-634.

73. **Eichwald, C., F. Vascotto, E. Fabbretti, and O. R. Burrone.** 2002. Rotavirus NSP5: mapping phosphorylation sites and kinase activation and viroplasm localization domains. J Virol **76:**3461-70.

74. **Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl.** 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411:**494-8.

75. **Enouf, V., S. Chwetzoff, G. Trugnan, and J. Cohen.** 2003. Interactions of rotavirus VP4 spike protein with the endosomal protein Rab5 and the prenylated Rab acceptor PRA1. J Virol **77:**7041-7.

76. **Ericson, B. L., D. Y. Graham, B. B. Mason, and M. K. Estes.** 1982. Identification, synthesis, and modifications of simian rotavirus SA11 polypeptides in infected cells. J Virol **42:**825-39.

77. **Erk, I., J. C. Huet, M. Duarte, S. Duquerroy, F. Rey, J. Cohen, and J. Lepault.** 2003. A zinc ion controls assembly and stability of the major capsid protein of rotavirus. J Virol **77:**3595-601.

78. **Estes, M.** 2001. Rotaviruses and their replication, p. 1747-1785. *In* P. Howley (ed.), Fields Virology, fourth ed, vol. 2. Lippincott Williams and Wilkins, New York.

79. **Estes, M. K., D. Y. Graham, C. P. Gerba, and E. M. Smith.** 1979. Simian rotavirus SA11 replication in cell cultures. J Virol **31:**810-5.

80. **Fabbretti, E., I. Afrikanova, F. Vascotto, and O. R. Burrone.** 1999. Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo. J Gen Virol **80 (Pt 2):**333-9.

81. **Fiore, L., H. B. Greenberg, and E. R. Mackow.** 1991. The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin. Virology **181:**553-63.

82. **Flores, J., K. Midthun, Y. Hoshino, K. Green, M. Gorziglia, A. Z. Kapikian, and R. M. Chanock.** 1986. Conservation of the fourth gene among rotaviruses recovered from asymptomatic newborn infants and its possible role in attenuation. J Virol **60:**972-9.

83. **Franco, M. A., and H. B. Greenberg.** 2000. Immunity to homologous rotavirus infection in adult mice. Trends Microbiol **8:**50-2.

84. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc Natl Acad Sci U S A **83:**8122-6.

85. **Fukuhara, N., K. Nishikawa, M. Gorziglia, and A. Z. Kapikian.** 1989. Nucleotide sequence of gene segment 1 of a porcine rotavirus strain. Virology **173:**743-9.

86. **Gajardo, R., P. Vende, D. Poncet, and J. Cohen.** 1997. Two proline residues are essential in the calcium-binding activity of rotavirus VP7 outer capsid protein. J Virol **71:**2211-6.

87. **Gallegos, C. O., and J. T. Patton.** 1989. Characterization of rotavirus replication intermediates: a model for the assembly of single-shelled particles. Virology **172:**616-27.

88. **Ge, Q., L. Filip, A. Bai, T. Nguyen, H. N. Eisen, and J. Chen.** 2004. Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci U S A **101:**8676-81.

89. **Gitlin, L., S. Karelsky, and R. Andino.** 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature **418:**430-4.

90. **Goh, P. Y., Y. J. Tan, S. P. Lim, Y. H. Tan, S. G. Lim, F. Fuller-Pace, and W. Hong.** 2004. Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. J Virol **78:**5288-98.

91. **Golantsova, N. E., E. E. Gorbunova, and E. R. Mackow.** 2004. Discrete domains within the rotavirus VP5* direct peripheral membrane association and membrane permeability. J Virol **78:**2037-44.

92. **Gonzalez, R. A., R. Espinosa, P. Romero, S. Lopez, and C. F. Arias.** 2000. Relative localization of viroplasmic and endoplasmic reticulum-resident rotavirus proteins in infected cells. Arch Virol **145:**1963-73.

93. **Gonzalez, R. A., M. A. Torres-Vega, S. Lopez, and C. F. Arias.** 1998. In vivo interactions among rotavirus nonstructural proteins. Arch Virol **143:**981-96.

94. **Gonzalez, S. A., and J. L. Affranchino.** 1995. Assembly of double-layered virus-like particles in mammalian cells by coexpression of human rotavirus VP2 and VP6. J Gen Virol **76 (Pt 9):**2357-60.

95. **Gonzalez, S. A., and O. R. Burrone.** 1991. Rotavirus NS26 is modified by addition of single O-linked residues of N-acetylglucosamine. Virology **182:**8-16.

96. **Gorziglia, M., Y. Hoshino, A. Buckler-White, I. Blumentals, R. Glass, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1986. Conservation of amino acid sequence of VP8 and cleavage region of 84-kDa outer capsid protein among rotaviruses recovered from asymptomatic neonatal infection. Proc Natl Acad Sci U S A **83:**7039-43.

97. **Gorziglia, M., C. Larrea, F. Liprandi, and J. Esparza.** 1985. Biochemical evidence for the oligomeric (possibly trimeric) structure of the major inner capsid polypeptide (45K) of rotaviruses. J Gen Virol **66 (Pt 9):**1889-900.

98. **Gouet, P., J. M. Diprose, J. M. Grimes, R. Malby, J. N. Burroughs, S. Zientara, D. I. Stuart, and P. P. Mertens.** 1999. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. Cell **97:**481-90.

99. **Graff, J. W., D. N. Mitzel, C. M. Weisend, M. L. Flenniken, and M. E. Hardy.** 2002. Interferon regulatory factor 3 is a cellular partner of rotavirus NSP1. J Virol **76:**9545-50.

100. **Graham, K. L., P. Halasz, Y. Tan, M. J. Hewish, Y. Takada, E. R. Mackow, M. K. Robinson, and B. S. Coulson.** 2003. Integrin-using rotaviruses bind alpha2beta1 integrin alpha2 I domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during cell entry. J Virol **77:**9969-78.

101. **Groft, C. M., and S. K. Burley.** 2002. Recognition of eIF4G by rotavirus NSP3 reveals a basis for mRNA circularization. Mol Cell **9:**1273-83.

102. **Guerrero, C. A., D. Bouyssounade, S. Zarate, P. Isa, T. Lopez, R. Espinosa, P. Romero, E. Mendez, S. Lopez, and C. F. Arias.** 2002. Heat shock cognate protein 70 is involved in rotavirus cell entry. J Virol **76:**4096-102.

103. **Guerrero, C. A., S. Zarate, G. Corkidi, S. Lopez, and C. F. Arias.** 2000. Biochemical characterization of rotavirus receptors in MA104 cells. J Virol **74:**9362-71.

104. **Haasnoot, P. C., D. Cupac, and B. Berkhout.** 2003. Inhibition of virus replication by RNA interference. J Biomed Sci **10:**607-16.

105. **Hart, G. W., K. D. Greis, L. Y. Dong, M. A. Blomberg, T. Y. Chou, M. S. Jiang, E. P. Roquemore, D. M. Snow, L. K. Kreppel, R. N. Cole, and et al.** 1995. O-linked N-acetylglucosamine: the "yin-yang" of Ser/Thr phosphorylation? Nuclear and cytoplasmic glycosylation. Adv Exp Med Biol **376:**115-23.

106. **Hewish, M. J., Y. Takada, and B. S. Coulson.** 2000. Integrins alpha2beta1 and alpha4beta1 can mediate SA11 rotavirus attachment and entry into cells. J Virol **74:**228-36.

107. **Hino, S., T. Michiue, M. Asashima, and A. Kikuchi.** 2003. Casein kinase I epsilon enhances the binding of Dvl-1 to Frat-1 and is essential for Wnt-3a-induced accumulation of beta-catenin. J Biol Chem **278:**14066-73.

108. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc Natl Acad Sci U S A **82:**8701-4.

109. **Hua, J., X. Chen, and J. T. Patton.** 1994. Deletion mapping of the rotavirus metalloprotein NS53 (NSP1): the conserved cysteine-rich region is essential for virus-specific RNA binding. J Virol **68:**3990-4000.

110. **Hua, J., and J. T. Patton.** 1994. The carboxyl-half of the rotavirus nonstructural protein NS53 (NSP1) is not required for virus replication. Virology **198:**567-76.

111. **Hui, E. K., E. M. Yap, D. S. An, I. S. Chen, and D. P. Nayak.** 2004. Inhibition of influenza virus matrix (M1) protein expression and virus replication by U6 promoter-driven and lentivirus-mediated delivery of siRNA. J Gen Virol **85:**1877-84.

112. **Hynes, R. O.** 2002. Integrins: bidirectional, allosteric signaling machines. Cell **110:**673-87.

113. **Iturriza-Gomara, M., D. Cubitt, D. Steele, J. Green, D. Brown, G. Kang, U. Desselberger, and J. Gray.** 2000. Characterisation of rotavirus G9 strains isolated in the UK between 1995 and 1998. J Med Virol **61:**510-7.

114. **Jayaram, H., M. K. Estes, and B. V. Prasad.** 2004. Emerging themes in rotavirus cell entry, genome organization, transcription and replication. Virus Res **101:**67-81.

115. **Jayaram, H., Z. Taraporewala, J. T. Patton, and B. V. Prasad.** 2002. Rotavirus protein involved in genome replication and packaging exhibits a HIT-like fold. Nature **417:**311-5.

116. **Jiang, M., and J. Milner.** 2005. Selective silencing of viral gene E6 and E7 expression in HPV-positive human cervical carcinoma cells using small interfering RNAs. Methods Mol Biol **292:**401-20.

117. **Jourdan, N., M. Maurice, D. Delautier, A. M. Quero, A. L. Servin, and G. Trugnan.** 1997. Rotavirus is released from the apical surface of cultured human intestinal cells through nonconventional vesicular transport that bypasses the Golgi apparatus. J Virol **71:**8268-78.

118. **Kabcenell, A. K., and P. H. Atkinson.** 1985. Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. J Cell Biol **101:**1270-80.

119. **Kabcenell, A. K., M. S. Poruchynsky, A. R. Bellamy, H. B. Greenberg, and P. H. Atkinson.** 1988. Two forms of VP7 are involved in assembly of SA11 rotavirus in endoplasmic reticulum. J Virol **62:**2929-41.

120. **Kaljot, K. T., R. D. Shaw, D. H. Rubin, and H. B. Greenberg.** 1988. Infectious rotavirus enters cells by direct cell membrane penetration, not by endocytosis. J Virol **62:**1136-44.

121. **Kapadia, S. B., A. Brideau-Andersen, and F. V. Chisari.** 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs. Proc Natl Acad Sci U S A **100:**2014-8.

122. **Kattoura, M. D., L. L. Clapp, and J. T. Patton.** 1992. The rotavirus nonstructural protein, NS35, possesses RNA-binding activity in vitro and in vivo. Virology **191:**698-708.

123. **Kearney, K., D. Chen, Z. F. Taraporewala, P. Vende, Y. Hoshino, M. A. Tortorici, M. Barro, and J. T. Patton.** 2004. Cell-line-induced mutation of the rotavirus genome alters expression of an IRF3-interacting protein. Embo J **23:**4072-81.

124. **Kirkwood, C. D., J. R. Gentsch, Y. Hoshino, H. F. Clark, and R. I. Glass.** 1999. Genetic and antigenic characterization of a serotype P[6]G9 human rotavirus strain isolated in the United States. Virology **256:**45-53.

125. **Knipe, D., C. E. Samuel, and P. Palese.** 2001. Virus-Host Interactions, p. 133-170. *In* P. Howley (ed.), Fields Virology, fourth ed, vol. 1. Lippincott Williams and Wilkins, New York.

126. **Knippschild, U., A. Gocht, S. Wolff, N. Huber, J. Lohler, and M. Stoter.** 2005. The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. Cell Signal **17:**675-89.

127. **Kohli, E., P. Pothier, G. Tosser, J. Cohen, A. M. Sandino, and E. Spencer.** 1993. In vitro reconstitution of rotavirus transcriptional activity using viral cores and recombinant baculovirus expressed VP6. Arch Virol **133:**451-8.

128. **Kovacs-Nolan, J., D. Yoo, and Y. Mine.** 2003. Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus. Biochem J **376:**269-75.

129. **Labbe, M., P. Baudoux, A. Charpilienne, D. Poncet, and J. Cohen.** 1994. Identification of the nucleic acid binding domain of the rotavirus VP2 protein. J Gen Virol **75 (Pt 12):**3423-30.

130. **Labbe, M., A. Charpilienne, S. E. Crawford, M. K. Estes, and J. Cohen.** 1991. Expression of rotavirus VP2 produces empty corelike particles. J Virol **65:**2946-52.

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

132. **LaMonica, R., S. S. Kocer, J. Nazarova, W. Dowling, E. Geimonen, R. D. Shaw, and E. R. Mackow.** 2001. VP4 differentially regulates TRAF2 signaling, disengaging JNK activation while directing NF-kappa B to effect rotavirus-specific cellular responses. J Biol Chem **276:**19889-96.

133. **Lawton, J. A., M. Estes, and B. V. Prasad.** 2000. Mechanism of genome transcription in segmented dsRNA viruses. Advances in virus research **55:**185-229.

134. **Lawton, J. A., M. K. Estes, and B. V. Prasad.** 1999. Comparative structural analysis of transcriptionally competent and incompetent rotavirus-antibody complexes. Proc Natl Acad Sci U S A **96:**5428-33.

135. **Lawton, J. A., M. K. Estes, and B. V. Prasad.** 2001. Identification and characterization of a transcription pause site in rotavirus. J Virol **75:**1632-42.

136. **Lawton, J. A., M. K. Estes, and B. V. Prasad.** 2000. Mechanism of genome transcription in segmented dsRNA viruses. Adv Virus Res **55:**185-229.

137. **Lawton, J. A., M. K. Estes, and B. V. Prasad.** 1997. Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. Nat Struct Biol **4:**118-21.

138. **Lawton, J. A., C. Q. Zeng, S. K. Mukherjee, J. Cohen, M. K. Estes, and B. V. Prasad.** 1997. Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: implications for the architecture of the VP2 capsid layer. J Virol **71:**7353-60.

139. **Lee, M. T., G. A. Coburn, M. O. McClure, and B. R. Cullen.** 2003. Inhibition of human immunodeficiency virus type 1 replication in primary macrophages by using Tat- or CCR5-specific small interfering RNAs expressed from a lentivirus vector. J Virol **77:**11964-72.

140. **Lepault, J., I. Petitpas, I. Erk, J. Navaza, D. Bigot, M. Dona, P. Vachette, J. Cohen, and F. A. Rey.** 2001. Structural polymorphism of the major capsid protein of rotavirus. Embo J **20:**1498-507.

141. **Li, Z. N., S. Hongo, K. Sugawara, K. Sugahara, E. Tsuchiya, Y. Matsuzaki, and K. Nakamura.** 2001. The sites for fatty acylation, phosphorylation and intermolecular disulphide bond formation of influenza C virus CM2 protein. J Gen Virol **82:**1085-93.

142. **Lima, C. D., M. G. Klein, and W. A. Hendrickson.** 1997. Structure-based analysis of catalysis and substrate definition in the HIT protein family. Science **278:**286-90.

143. **Liu, C., Y. Li, M. Semenov, C. Han, G. H. Baeg, Y. Tan, Z. Zhang, X. Lin, and X. He.** 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell **108:**837-47.

144. **Liu, M., and M. K. Estes.** 1989. Nucleotide sequence of the simian rotavirus SA11 genome segment 3. Nucleic Acids Res **17:**7991.

145. **Liu, M., N. M. Mattion, and M. K. Estes.** 1992. Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. Virology **188:**77-84.

146. **Lopez, S., C. F. Arias, J. R. Bell, J. H. Strauss, and R. T. Espejo.** 1985. Primary structure of the cleavage site associated with trypsin enhancement of rotavirus SA11 infectivity. Virology **144:**11-9.

147. **Lopez, T., M. Camacho, M. Zayas, R. Najera, R. Sanchez, C. F. Arias, and S. Lopez.** 2005. Silencing the morphogenesis of rotavirus. J Virol **79:**184-92.

148. **Lopez, T., M. Rojas, C. Ayala-Breton, S. Lopez, and C. F. Arias.** 2005. Reduced expression of the rotavirus NSP5 gene has a pleiotropic effect on virus replication. J Gen Virol **86:**1609-17.

149. **Ludert, J. E., F. Michelangeli, F. Gil, F. Liprandi, and J. Esparza.** 1987. Penetration and uncoating of rotaviruses in cultured cells. Intervirology **27:**95-101.

150. **Lundgren, O., A. T. Peregrin, K. Persson, S. Kordasti, I. Uhnoo, and L. Svensson.** 2000. Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhea. Science **287:**491-5.

151. **Maass, D. R., and P. H. Atkinson.** 1994. Retention by the endoplasmic reticulum of rotavirus VP7 is controlled by three adjacent amino-terminal residues. J Virol **68:**366-78.

152. **Maass, D. R., and P. H. Atkinson.** 1990. Rotavirus proteins VP7, NS28, and VP4 form oligomeric structures. J Virol **64:**2632-41.

153. **Mackow, E. R., R. D. Shaw, S. M. Matsui, P. T. Vo, M. N. Dang, and H. B. Greenberg.** 1988. The rhesus rotavirus gene encoding protein VP3: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region. Proc Natl Acad Sci U S A **85:**645-9.

154. **Manel, N., F. J. Kim, S. Kinet, N. Taylor, M. Sitbon, and J. L. Battini.** 2003. The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. Cell **115:**449-59.

155. **Mansell, E. A., and J. T. Patton.** 1990. Rotavirus RNA replication: VP2, but not VP6, is necessary for viral replicase activity. J Virol **64:**4988-96.

156. **Martincic, I., M. E. Peralta, and J. K. Ngsee.** 1997. Isolation and characterization of a dual prenylated Rab and VAMP2 receptor. J Biol Chem **272:**26991-8.

157. **Mathieu, M., I. Petitpas, J. Navaza, J. Lepault, E. Kohli, P. Pothier, B. V. Prasad, J. Cohen, and F. A. Rey.** 2001. Atomic structure of the major capsid protein of rotavirus: implications for the architecture of the virion. Embo J **20:**1485-97.

158. **Mattion, N. M., J. Cohen, C. Aponte, and M. K. Estes.** 1992. Characterization of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34. Virology **190:**68-83.

159. **Mattion, N. M., D. B. Mitchell, G. W. Both, and M. K. Estes.** 1991. Expression of rotavirus proteins encoded by alternative open reading frames of genome segment 11. Virology **181:**295-304.

160. **McCaffrey, A. P., L. Meuse, T. T. Pham, D. S. Conklin, G. J. Hannon, and M. A. Kay.** 2002. RNA interference in adult mice. Nature **418:**38-9.

161. **McCrae, M. A., and G. P. Faulkner-Valle.** 1981. Molecular biology of rotaviruses. I. Characterization of basic growth parameters and pattern of macromolecular synthesis. J Virol **39:**490-6.

162. **Michelangeli, F., F. Liprandi, M. E. Chemello, M. Ciarlet, and M. C. Ruiz.** 1995. Selective depletion of stored calcium by thapsigargin blocks rotavirus maturation but not the cytopathic effect. J Virol **69:**3838-47.

163. **Mirazimi, A., M. Nilsson, and L. Svensson.** 1998. The molecular chaperone calnexin interacts with the NSP4 enterotoxin of rotavirus in vivo and in vitro. J Virol **72:**8705-9.

164. **Mirazimi, A., and L. Svensson.** 2000. ATP is required for correct folding and disulfide bond formation of rotavirus VP7. J Virol **74:**8048-52.

165. **Mitchell, D. B., and G. W. Both.** 1990. Conservation of a potential metal binding motif despite extensive sequence diversity in the rotavirus nonstructural protein NS53. Virology **174:**618-21.

166. **Modrof, J., K. Lymperopoulos, and P. Roy.** 2005. Phosphorylation of bluetongue virus nonstructural protein 2 is essential for formation of viral inclusion bodies. J Virol **79:**10023-31.

167. **Mohan, K. V., and C. D. Atreya.** 2001. Nucleotide sequence analysis of rotavirus gene 11 from two tissue culture-adapted ATCC strains, RRV and Wa. Virus Genes **23:**321-9.

168. **Mohan, K. V., J. Muller, and C. D. Atreya.** 2003. The N- and C-terminal regions of rotavirus NSP5 are the critical determinants for the formation of viroplasm-like structures independent of NSP2. J Virol **77:**12184-92.

169. **Morris, A. P., and M. K. Estes.** 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. VIII. Pathological consequences of rotavirus infection and its enterotoxin. Am J Physiol Gastrointest Liver Physiol **281:**G303-10.

170. **Morris, A. P., J. K. Scott, J. M. Ball, C. Q. Zeng, W. K. O'Neal, and M. K. Estes.** 1999. NSP4 elicits age-dependent diarrhea and Ca(2+)mediated I(-) influx into intestinal crypts of CF mice. Am J Physiol **277:**G431-44.

171. **Nejmeddine, M., G. Trugnan, C. Sapin, E. Kohli, L. Svensson, S. Lopez, and J. Cohen.** 2000. Rotavirus spike protein VP4 is present at the plasma membrane and is associated with microtubules in infected cells. J Virol **74:**3313-20.

172. **O'Brien, G. J., C. J. Bryant, C. Voogd, H. B. Greenberg, R. C. Gardner, and A. R. Bellamy.** 2000. Rotavirus VP6 expressed by PVX vectors in Nicotiana benthamiana coats PVX rods and also assembles into viruslike particles. Virology **270:**444-53.

173. **O'Brien, J. A., J. A. Taylor, and A. R. Bellamy.** 2000. Probing the structure of rotavirus NSP4: a short sequence at the extreme C terminus mediates binding to the inner capsid particle. J Virol **74:**5388-94.

174. **Offit, P. A., and G. Blavat.** 1986. Identification of the two rotavirus genes determining neutralization specificities. J Virol **57:**376-8.

175. **Oka, T., T. Nakagomi, and O. Nakagomi.** 2000. Apparent re-emergence of serotype G9 in 1995 among rotaviruses recovered from Japanese children hospitalized with acute gastroenteritis. Microbiol Immunol **44:**957-61.

176. **Palombo, E. A., P. J. Masendycz, H. C. Bugg, N. Bogdanovic-Sakran, G. L. Barnes, and R. F. Bishop.** 2000. Emergence of serotype G9 human rotaviruses in Australia. J Clin Microbiol **38:**1305-6.

177. **Parashar, U. D., E. G. Hummelman, J. S. Bresee, M. A. Miller, and R. I. Glass.** 2003. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis **9:**565-72.

178. **Patton, J. T.** 1996. Rotavirus VP1 alone specifically binds to the 3' end of viral mRNA, but the interaction is not sufficient to initiate minus-strand synthesis. J Virol **70:**7940-7.

179. **Patton, J. T.** 1995. Structure and function of the rotavirus RNA-binding proteins. J Gen Virol **76 (Pt 11):**2633-44.

180. **Patton, J. T., and D. Chen.** 1999. RNA-binding and capping activities of proteins in rotavirus open cores. J Virol **73:**1382-91.

181. **Patton, J. T., and C. O. Gallegos.** 1990. Rotavirus RNA replication: single-stranded RNA extends from the replicase particle. J Gen Virol **71 (Pt 5):**1087-94.

182. **Patton, J. T., and C. O. Gallegos.** 1988. Structure and protein composition of the rotavirus replicase particle. Virology **166:**358-65.

183. **Patton, J. T., and E. Spencer.** 2000. Genome replication and packaging of segmented double-stranded RNA viruses. Virology **277:**217-25.

184. **Patton, J. T., M. Wentz, J. Xiaobo, and R. F. Ramig.** 1996. cis-Acting signals that promote genome replication in rotavirus mRNA. J Virol **70:**3961-71.

185. **Pesavento, J. B., S. E. Crawford, E. Roberts, M. K. Estes, and B. V. Prasad.** 2005. pH-induced conformational change of the rotavirus VP4 spike: implications for cell entry and antibody neutralization. J Virol **79:**8572-80.

186. **Pesavento, J. B., J. A. Lawton, M. E. Estes, and B. V. Venkataram Prasad.** 2001. The reversible condensation and expansion of the rotavirus genome. Proc Natl Acad Sci U S A **98:**1381-6.

187. **Petrie, B. L., M. K. Estes, and D. Y. Graham.** 1983. Effects of tunicamycin on rotavirus morphogenesis and infectivity. J Virol **46:**270-4.

188. **Petrie, B. L., H. B. Greenberg, D. Y. Graham, and M. K. Estes.** 1984. Ultrastructural localization of rotavirus antigens using colloidal gold. Virus Res **1:**133-52.

189. **Pinto, R. M., J. M. Diez, and A. Bosch.** 1994. Use of the colonic carcinoma cell line CaCo-2 for in vivo amplification and detection of enteric viruses. J Med Virol **44:**310-5.

190. **Piron, M., P. Vende, J. Cohen, and D. Poncet.** 1998. Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. Embo J **17:**5811-21.

191. **Pizarro, J. L., A. M. Sandino, J. M. Pizarro, J. Fernandez, and E. Spencer.** 1991. Characterization of rotavirus guanylyltransferase activity associated with polypeptide VP3. J Gen Virol **72 (Pt 2):**325-32.

192. **Pizarro, J. M., J. L. Pizarro, J. Fernandez, A. M. Sandino, and E. Spencer.** 1991. Effect of nucleotide analogues on rotavirus transcription and replication. Virology **184:**768-72.

193. **Poncet, D., C. Aponte, and J. Cohen.** 1993. Rotavirus protein NSP3 (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells. J Virol **67:**3159-65.

194. **Poncet, D., S. Laurent, and J. Cohen.** 1994. Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. Embo J **13:**4165-73.

195. **Poncet, D., P. Lindenbaum, R. L'Haridon, and J. Cohen.** 1997. In vivo and in vitro phosphorylation of rotavirus NSP5 correlates with its localization in viroplasms. J Virol **71:**34-41.

196. **Poruchynsky, M. S., and P. H. Atkinson.** 1991. Rotavirus protein rearrangements in purified membrane-enveloped intermediate particles. J Virol **65:**4720-7.

197. **Poruchynsky, M. S., D. R. Maass, and P. H. Atkinson.** 1991. Calcium depletion blocks the maturation of rotavirus by altering the oligomerization of virus-encoded proteins in the ER. J Cell Biol **114:**651-6.

198. **Poruchynsky, M. S., C. Tyndall, G. W. Both, F. Sato, A. R. Bellamy, and P. H. Atkinson.** 1985. Deletions into an NH2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. J Cell Biol **101:**2199-209.

199. **Prasad, B. V., J. W. Burns, E. Marietta, M. K. Estes, and W. Chiu.** 1990. Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. Nature **343:**476-9.

200. **Prasad, B. V., and M. Estes.** 1997. Molecular Basis of Rotavirus Replication. Structure-Function Correlations., p. 239-268. *In* G. R.L. (ed.), Structural Biology of Viruses. Oxford University Press, New York.

201. **Prasad, B. V., R. Rothnagel, C. Q. Zeng, J. Jakana, J. A. Lawton, W. Chiu, and M. K. Estes.** 1996. Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. Nature **382:**471-3.

202. **Prasad, B. V., G. J. Wang, J. P. Clerx, and W. Chiu.** 1988. Three-dimensional structure of rotavirus. J Mol Biol **199:**269-75.

203. **Pulgar, V., O. Marin, F. Meggio, C. C. Allende, J. E. Allende, and L. A. Pinna.** 1999. Optimal sequences for non-phosphate-directed phosphorylation by protein kinase CK1 (casein kinase-1)--a re-evaluation. Eur J Biochem **260:**520-6.

204. **Ramachandran, M., B. K. Das, A. Vij, R. Kumar, S. S. Bhambal, N. Kesari, H. Rawat, L. Bahl, S. Thakur, P. A. Woods, R. I. Glass, M. K. Bhan, and J. R. Gentsch.** 1996. Unusual diversity of human rotavirus G and P genotypes in India. J Clin Microbiol **34:**436-9.

205. **Ramig, R. F., and B. L. Petrie.** 1984. Characterization of temperature-sensitive mutants of simian rotavirus SA11: protein synthesis and morphogenesis. J Virol **49:**665-73.

206. **Ready, K. F., and M. Sabara.** 1987. In vitro assembly of bovine rotavirus nucleocapsid protein. Virology **157:**189-98.

207. **Ruiz, M. C., M. J. Abad, A. Charpilienne, J. Cohen, and F. Michelangeli.** 1997. Cell lines susceptible to infection are permeabilized by cleaved and solubilized outer layer proteins of rotavirus. J Gen Virol **78 (Pt 11):**2883-93.

208. **Ruiz, M. C., A. Charpilienne, F. Liprandi, R. Gajardo, F. Michelangeli, and J. Cohen.** 1996. The concentration of Ca2+ that solubilizes outer capsid proteins from rotavirus particles is dependent on the strain. J Virol **70:**4877-83.

209. **Ruiz, M. C., J. Cohen, and F. Michelangeli.** 2000. Role of Ca2+in the replication and pathogenesis of rotavirus and other viral infections. Cell Calcium **28:**137-49.

210. **Sabara, M., L. A. Babiuk, J. Gilchrist, and V. Misra.** 1982. Effect of tunicamycin on rotavirus assembly and infectivity. J Virol **43:**1082-90.

211. **Sabara, M., K. F. Ready, P. J. Frenchick, and L. A. Babiuk.** 1987. Biochemical evidence for the oligomeric arrangement of bovine rotavirus nucleocapsid protein and its possible significance in the immunogenicity of this protein. J Gen Virol **68 (Pt 1):**123-33.

212. **Sanchez-San Martin, C., T. Lopez, C. F. Arias, and S. Lopez.** 2004. Characterization of rotavirus cell entry. J Virol **78:**2310-8.

213. **Sandino, A. M., J. Fernandez, J. Pizarro, M. Vasquez, and E. Spencer.** 1994. Structure of rotavirus particle: interaction of the inner capsid protein VP6 with the core polypeptide VP3. Biol Res **27:**39-48.

214. **Santos, N., E. M. Volotao, C. C. Soares, M. C. Albuquerque, F. M. da Silva, V. Chizhikov, and Y. Hoshino.** 2003. VP7 gene polymorphism of serotype G9 rotavirus strains and its impact on G genotype determination by PCR. Virus Res **93:**127-38.

215. **Sapin, C., O. Colard, O. Delmas, C. Tessier, M. Breton, V. Enouf, S. Chwetzoff, J. Ouanich, J. Cohen, C. Wolf, and G. Trugnan.** 2002. Rafts promote assembly and atypical targeting of a nonenveloped virus, rotavirus, in Caco-2 cells. J Virol **76:**4591-602.

216. **Schuck, P., Z. Taraporewala, P. McPhie, and J. T. Patton.** 2001. Rotavirus nonstructural protein NSP2 self-assembles into octamers that undergo ligand-induced conformational changes. J Biol Chem **276:**9679-87.

217. **Schwartz-Cornil, I., Y. Benureau, H. Greenberg, B. A. Hendrickson, and J. Cohen.** 2002. Heterologous protection induced by the inner capsid proteins of rotavirus requires transcytosis of mucosal immunoglobulins. J Virol **76:**8110-7.

218. **Sen, A., R. Steele, A. K. Ghosh, A. Basu, R. Ray, and R. B. Ray.** 2003. Inhibition of hepatitis C virus protein expression by RNA interference. Virus Res **96:**27-35.

219. **Seo, M. Y., S. Abrignani, M. Houghton, and J. H. Han.** 2003. Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. J Virol **77:**810-2.

220. **Shaw, A. L., R. Rothnagel, D. Chen, R. F. Ramig, W. Chiu, and B. V. Prasad.** 1993. Three-dimensional visualization of the rotavirus hemagglutinin structure. Cell **74:**693-701.

221. **Silvestri, L. S., Z. F. Taraporewala, and J. T. Patton.** 2004. Rotavirus replication: plus-sense templates for double-stranded RNA synthesis are made in viroplasms. J Virol **78:**7763-74.

222. **Sledz, C. A., M. Holko, M. J. de Veer, R. H. Silverman, and B. R. Williams.** 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol **5:**834-9.

223. **Spencer, E., and M. L. Arias.** 1981. In vitro transcription catalyzed by heat-treated human rotavirus. J Virol **40:**1-10.

224. **Stacy-Phipps, S., and J. T. Patton.** 1987. Synthesis of plus- and minus-strand RNA in rotavirus-infected cells. J Virol **61:**3479-84.

225. **Stirzaker, S. C., P. L. Whitfeld, D. L. Christie, A. R. Bellamy, and G. W. Both.** 1987. Processing of rotavirus glycoprotein VP7: implications for the retention of the protein in the endoplasmic reticulum. J Cell Biol **105:**2897-903.

226. **Suzuki, H., S. Kitaoka, T. Konno, T. Sato, and N. Ishida.** 1985. Two modes of human rotavirus entry into MA 104 cells. Arch Virol **85:**25-34.

227. **Tafazoli, F., C. Q. Zeng, M. K. Estes, K. E. Magnusson, and L. Svensson.** 2001. NSP4 enterotoxin of rotavirus induces paracellular leakage in polarized epithelial cells. J Virol **75:**1540-6.

228. **Tan, F. L., and J. Q. Yin.** 2004. RNAi, a new therapeutic strategy against viral infection. Cell Res **14:**460-6.

229. **Taraporewala, Z., D. Chen, and J. T. Patton.** 1999. Multimers formed by the rotavirus nonstructural protein NSP2 bind to RNA and have nucleoside triphosphatase activity. J Virol **73:**9934-43.

230. **Taraporewala, Z. F., and J. T. Patton.** 2001. Identification and characterization of the helix-destabilizing activity of rotavirus nonstructural protein NSP2. J Virol **75:**4519-27.

231. **Taraporewala, Z. F., and J. T. Patton.** 2004. Nonstructural proteins involved in genome packaging and replication of rotaviruses and other members of the Reoviridae. Virus Res **101:**57-66.

232. **Taraporewala, Z. F., P. Schuck, R. F. Ramig, L. Silvestri, and J. T. Patton.** 2002. Analysis of a temperature-sensitive mutant rotavirus indicates that NSP2 octamers are the functional form of the protein. J Virol **76:**7082-93.

233. **Taylor, J. A., J. A. O'Brien, and M. Yeager.** 1996. The cytoplasmic tail of NSP4, the endoplasmic reticulum-localized non-structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. Embo J **15:**4469-76.

234. **Thouvenin, E., G. Schoehn, F. Rey, I. Petitpas, M. Mathieu, M. C. Vaney, J. Cohen, E. Kohli, P. Pothier, and E. Hewat.** 2001. Antibody inhibition of the transcriptase activity of the rotavirus DLP: a structural view. J Mol Biol **307:**161-72.

235. **Torres-Vega, M. A., R. A. Gonzalez, M. Duarte, D. Poncet, S. Lopez, and C. F. Arias.** 2000. The C-terminal domain of rotavirus NSP5 is essential for its multimerization, hyperphosphorylation and interaction with NSP6. J Gen Virol **81:**821-30.

236. **Tortorici, M. A., T. J. Broering, M. L. Nibert, and J. T. Patton.** 2003. Template recognition and formation of initiation complexes by the replicase of a segmented double-stranded RNA virus. J Biol Chem **278:**32673-82.

237. **Tosser, G., M. Labbe, M. Bremont, and J. Cohen.** 1992. Expression of the major capsid protein VP6 of group C rotavirus and synthesis of chimeric single-shelled particles by using recombinant baculoviruses. J Virol **66:**5825-31.

238. **Unicomb, L. E., G. Podder, J. R. Gentsch, P. A. Woods, K. Z. Hasan, A. S. Faruque, M. J. Albert, and R. I. Glass.** 1999. Evidence of high-frequency genomic reassortment of group A rotavirus strains in Bangladesh: emergence of type G9 in 1995. J Clin Microbiol **37:**1885-91.

239. **Valenzuela, S., J. Pizarro, A. M. Sandino, M. Vasquez, J. Fernandez, O. Hernandez, J. Patton, and E. Spencer.** 1991. Photoaffinity labeling of rotavirus VP1 with 8-azido-ATP: identification of the viral RNA polymerase. J Virol **65:**3964-7.

240. **Vascotto, F., M. Campagna, M. Visintin, A. Cattaneo, and O. R. Burrone.** 2004. Effects of intrabodies specific for rotavirus NSP5 during the virus replicative cycle. J Gen Virol **85:**3285-90.

241. **Vascotto, F., M. Visintin, A. Cattaneo, and O. R. Burrone.** 2005. Design and selection of an intrabody library produced de-novo for the non-structural protein NSP5 of rotavirus. J Immunol Methods **301:**31-40.

242. **Vende, P., M. Piron, N. Castagne, and D. Poncet.** 2000. Efficient translation of rotavirus mRNA requires simultaneous interaction of NSP3 with the eukaryotic translation initiation factor eIF4G and the mRNA 3' end. J Virol **74:**7064-71.

243. **Vende, P., Z. F. Taraporewala, and J. T. Patton.** 2002. RNA-binding activity of the rotavirus phosphoprotein NSP5 includes affinity for double-stranded RNA. J Virol **76:**5291-9.

244. **Vende, P., M. A. Tortorici, Z. F. Taraporewala, and J. T. Patton.** 2003. Rotavirus NSP2 interferes with the core lattice protein VP2 in initiation of minus-strand synthesis. Virology **313:**261-73.

245. **Vitour, D., P. Lindenbaum, P. Vende, M. M. Becker, and D. Poncet.** 2004. RoXaN, a novel cellular protein containing TPR, LD, and zinc finger motifs, forms a ternary complex with eukaryotic initiation factor 4G and rotavirus NSP3. J Virol **78:**3851-62.

246. **Wentz, M. J., J. T. Patton, and R. F. Ramig.** 1996. The 3'-terminal consensus sequence of rotavirus mRNA is the minimal promoter of negative-strand RNA synthesis. J Virol **70:**7833-41.

247. **Whitfeld, P. L., C. Tyndall, S. C. Stirzaker, A. R. Bellamy, and G. W. Both.** 1987. Location of sequences within rotavirus SA11 glycoprotein VP7 which direct it to the endoplasmic reticulum. Mol Cell Biol **7:**2491-7.

248. **WHO.** 1997. Report from consensus workshop: rotavirus vaccines for the immunization of children in developing countries. Geneva: Whorld Health Organisation.

249. **Widdowson, M. A., G. J. van Doornum, W. H. van der Poel, A. S. de Boer, U. Mahdi, and M. Koopmans.** 2000. Emerging group-A rotavirus and a nosocomial outbreak of diarrhoea. Lancet **356:**1161-2.

250. **Xu, A., A. R. Bellamy, and J. A. Taylor.** 2000. Immobilization of the early secretory pathway by a virus glycoprotein that binds to microtubules. Embo J **19:**6465-74.

251. **Yeager, M., J. A. Berriman, T. S. Baker, and A. R. Bellamy.** 1994. Three-dimensional structure of the rotavirus haemagglutinin VP4 by cryo-electron microscopy and difference map analysis. Embo J **13:**1011-8.

252. **Yeager, M., K. A. Dryden, N. H. Olson, H. B. Greenberg, and T. S. Baker.** 1990. Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. J Cell Biol **110:**2133-44.

253. **Yolken, R., S. Arango-Jaramillo, J. Eiden, and S. Vonderfecht.** 1988. Lack of genomic reassortment following infection of infant rats with group A and group B rotaviruses. J Infect Dis **158:**1120-3.

254. **Yoshinouchi, M., T. Yamada, M. Kizaki, J. Fen, T. Koseki, Y. Ikeda, T. Nishihara, and K. Yamato.** 2003. In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. Mol Ther **8:**762-8.

255. **Zarate, S., M. A. Cuadras, R. Espinosa, P. Romero, K. O. Juarez, M. Camacho-Nuez, C. F. Arias, and S. Lopez.** 2003. Interaction of rotaviruses with Hsc70 during cell entry is mediated by VP5. J Virol **77:**7254-60.

256. **Zarate, S., P. Romero, R. Espinosa, C. F. Arias, and S. Lopez.** 2004. VP7 mediates the interaction of rotaviruses with integrin alphavbeta3 through a novel integrin-binding site. J Virol **78:**10839-47.

257. **Zeng, C. Q., M. K. Estes, A. Charpilienne, and J. Cohen.** 1998. The N terminus of rotavirus VP2 is necessary for encapsidation of VP1 and VP3. J Virol **72:**201-8.

258. **Zeng, C. Q., M. J. Wentz, J. Cohen, M. K. Estes, and R. F. Ramig.** 1996. Characterization and replicase activity of double-layered and single-layered rotavirus-like particles expressed from baculovirus recombinants. J Virol **70:**2736-42.

259. **Zhang, M., C. Q. Zeng, Y. Dong, J. M. Ball, L. J. Saif, A. P. Morris, and M. K. Estes.** 1998. Mutations in rotavirus nonstructural glycoprotein NSP4 are associated with altered virus virulence. J Virol **72:**3666-72.

260. **Zhang, M., C. Q. Zeng, A. P. Morris, and M. K. Estes.** 2000. A functional NSP4 enterotoxin peptide secreted from rotavirus-infected cells. J Virol **74:**11663-70.

261. **Zhang, W., R. Singam, G. Hellermann, X. Kong, H. S. Juan, R. F. Lockey, S. J. Wu, K. Porter, and S. S. Mohapatra.** 2004. Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. Genet Vaccines Ther **2:**8.

262. **Zhou, Y., J. Supawadee, C. Khamwan, S. Tonusin, S. Peerakome, B. Kim, K. Kaneshi, Y. Ueda, S. Nakaya, K. Akatani, N. Maneekarn, and H. Ushijima.** 2001. Characterization of human rotavirus serotype G9 isolated in Japan and Thailand from 1995 to 1997. J Med Virol **65:**619-28.

Acknowledgements

Ci sono moltissime persone che desidero ringraziare. Prima di tutto 'el jefe' Oscar per la sua fiducia, il suo entusiasmo e per la sua grandissima disponibilita' ogni qual volta sono andata a bussare alla sua porta a chiedere consiglio. Inoltre vorrei ringraziare Mauricio (il Budini) del laboratorio di Jorge Allende con il quale ho collaborato con grande piacere per la realizzazione di una parte di questa tesi.
Il mio pensiero poi va ovviamente al Rotagroup. In particolare a Fulvia e Cathy che mi hanno accolto in questo laboratorio per le continue discussioni di lavoro (e non) avute negli anni di 'convivenza' in laboratorio. Ma ovviamente non posso dimenticare neanche i nuovi rotas, Bartek (il rotaboy), sempre gentilissimo disponibile e Francesca, la mia compagna di bancone 'gomito a gomito' per le continue consulenze di lavoro, di vita e per le virgole...
Ovviamente voglio poi menzionare tutte le altre persone del laboratorio che quasi subito, dopo il mio arrivo, sono diventate da colleghe amiche rendendo molto piu' allegra la convivenza quotidiana. In particolare 'la sister' Michela, amica e confidente, la sempre energetica Roby, ma anche Elisa , Fede (con Pollo), Mirza (il mio 'back to back colleague' in questi 4 anni), Marco, Edo, Monica, Alejandro ed ovviamente gli amici ormai lontani ma sempre presenti (chilenos de corazon) Pao e Jorge.
Ma il mio ringraziamento va anche a tutte quelle persone che, pur non essendo del lab, sono state compagne di vita, lavorativa e non, in questi anni. Prima di tutto le ragazze vecchie e nuove di via Berchet: Vale, la coinqui Carlotta, che mi sopporta in casa da ormai due anni (o forse e' il contrario?), Aga, Agi. Ed oltre a loro Milica compagna di lunghe, lunghissime chiacchierate, Kako, Pablo e tra i 'nuovi arrivi' in particolare Mohamed, Noor, Natasha, Lucia, Nabil.
Un ringraziamento speciale va poi ai miei due computer men (nonche' amici), quello storico di Carrara, Alessandro per la sua sempre perfetta consulenza in linea (telefonica) e quello di Trieste, Johan, sempre pronto, per il modico prezzo di una cena, a venirmi (spesso) a aiutare con il computer.
Vorrei poi citare le amiche carrarine, sempre disponibili quando avevo bisogno di sfogarmi sui miei problemi 'triestini', Paola, Annalisa, Chiara ed Elena.
Ed infine mia madre sempre presente anche nella distanza per il suo continuo appoggio ed aiuto ogni qualvolta io ne abbia avuto bisogno.