# "IDENTIFICATION AND CHARACTERIZATION OF AN ATPASE ACTIVITY ASSOCIATED WITH THE ROTAVIRUS PHOSPHOPROTEIN NSP5"

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by

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**FACULTAD DE CIENCIAS**

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# INFORME DE APROBACION

# TESIS DE DOCTORADO

Se informa a la Comisión de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado Presentada por la candidata:

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*It is the discovery of your wonderful existence that gave me the reason to wake up, to go on and exceed myself hopping to become better... May these words and my mirrors show you how much of your essence is in here and in every dream and image that shapes my life from the day I met you and on.*

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## ABREVIATIONS

| | |
|---|---|
| Ala: | Alanine |
| Arg: | Arginine |
| ATPase: | Adenosine triphosphate hydrolase. |
| AU: | Analytical Ultracentrifugation |
| CKI: | Casein Kinase I. |
| CKII: | Casein Kinase II. |
| C-Terminal: | Carboxy terminal. |
| DLP: | Double Layer Particle. |
| dsRNA: | Double Stranded RNA. |
| EDTA: | Ethylendiaminetetraacetic acid. |
| His: | Histidine. |
| IPTG: | Isopropyl-ß-D-thiogalactopyranosid. |
| LSB: | Low Salt Buffer. |
| Lys: | Lysine. |
| *mt:* | Mutant protein. |
| NDP: | Nucleotide diphosphate. |
| NSP: | Non structural protein. |
| N-Terminal: | Amino terminal. |
| NTP: | Nucleotide triphosphate. |
| NTPase: | Nucleotide triphosphate hydrolase. |
| $OD_{600}$: | Optical density at 600 nm. |

| | |
|---|---|
| ORF: | Open Reading Frame. |
| PAGE: | Polyacrylamide Gel of Electrophoresis. |
| PCR: | Polymerase Chain Reaction. |
| PVDF: | Polyvinylidene difluoride membrane. |
| RIs: | Replication intermediates. |
| RTPase: | RNA 5' triphosphatase. |
| SDS: | Sodium Dodecyl Sulfate. |
| ssRNA: | Single Stranded RNA. |
| SVPs: | Sub viral particles. |
| TLC: | Thin Layer Chromatography. |
| TLP: | Triple Layer Particle. |
| *ts* mutant: | Temperature sensitive mutant. |
| UTR: | Untranslated region. |
| VP: | Viral Protein. |
| *wt*: | Wild type. |

## SUMMARY

Rotaviruses, members of the *Reoviridae*, cause a severe gastroenteritis in young of many species. It has a devastating effect in third world countries, killing around half a million infants every year.

Although rotaviruses replication takes place in the cytoplasm, the viral genome, 11 segments of double stranded RNA (dsRNA), seems to be concealed from the cell defense systems thanks to the viroplasms. Viroplasms are electrodense cytoplasmic structures, which are visible in the infected cell shortly after the entrance of the virus particle (3 to 4 hours post infection). Their formation is essential for replication and viruses that fail to form these structures do not produce infective particles. The scaffold of viroplasm structures is made up from two non structural proteins, NSP2 and NSP5.

NSP2 is a basic protein of 35 kDa whose structure has been solved through x-ray crystallography. NSP2 has been characterized as a dsRNA helix-destabilizing protein as well as an NTPase and an RTPase.

NSP5 is codified by the smallest segment of rotavirus, gene 11. It is a dimeric protein of 198 amino acids, slightly acidic, with a sequence rich in serines and threonines.

NSP5 has an RNA binding activity for both, ssRNA and dsRNA, in a sequence unspecific manner. It has been also characterized as a phosphoprotein, phosphorylated and hyperphosphorylated *in vivo* during infection and when co-transfected with NSP2, but not hyperphosphorylated when transfected alone.

It has been proposed that NSP5 has an intrinsic autokinase activity positively affected by the presence of NSP2. When NSP2 is present, NSP5 is hyperphosphorylated *in vitro*, a phosphorylation that renders a different pattern of migration in comparison to the hyperphosphorylation pattern of the protein *in vivo*. The NTPase activity of NSP2 was shown to affect the autokinase activity of NSP5 *in vitro*, but *in vivo* there was no further effect of this mutations neither on the phosphorylation of NSP5 nor on viroplasm formation.

In this work a new enzymatic activity of NSP5 was identified, an ATPase activity. The characterization of the reaction of hydrolysis allowed the determination of the most convenient conditions for the hydrolysis assays. Recombinant purified NSP5 produced in bacteria was found to hydrolyze ATP in the β-γ phosphodiester bond. The hydrolysis only took place when a divalent cation was present, preferentially $Mg^{+2}$. Other nucleotides were not hydrolyzed to the same extent as ATP by NSP5.

Neither sequence specific nor random sequence single stranded RNA (ssRNA) had an effect on the ATP hydrolysis of NSP5. Double stranded RNA (dsRNA) had a mild positive effect on the hydrolysis of ATP when incubated with NSP5.

In contrast to the *in vitro* autophosphorylation of NSP5 that is induced by NSP2, the ATPase activity of NSP5 was neither affected neither by the presence of NSP2 nor by its NTPase activity.

Molecular simulation techniques allowed proposing a model of NSP5 structure and the critical aminoacids for ATP and $Mg^{+2}$ binding. Several amino acids suggested as critical by the molecular model were mutated through site directed mutagenesis and no

significant effect on the ATPase activity of NSP5 or on its phosphorylation *in vitro* was detected.

Recent reports have proposed a serine residue of NSP5, S67, to be phosphorylated *in vivo* by a cellular kinase, CKI, in the presence of NSP2, rendering the protein ready for further phosphorylations *in vivo*. In spite of the model proposed in which serine 67 plays an important role in phosphorylation of NSP5 *in vivo*, site directed mutagenesis of serine 67 did not have an effect either on the phosphorylation of the protein *in vitro* or on the ATPase activity of NSP5.

Thus, several evidences suggest the phosphorylation and hyperphosphorylation *in vivo* is not directly related to the ATP hydrolysis and the phosphorylation of NSP5 *in vitro*.

Sequences of NSP5 and NSP2 obtained either from laboratory strains of different virus genotypes and field samples of infected patients were analyzed. The sequences were highly conserved, in particular the sequence of NSP5. The sequences of NSP2 and NSP5 showed a similar distribution among strains when their phylogenetic analysis was compared but no clear correlation between the changes in NSP5 and those on the sequence of NSP2 in the different strains analyzed was detected. Also a correlation between the sources of the sequences, whether from a patient or from a laboratory strain, and the maintenance of the second open reading frame was detected.

## RESUMEN

Rotavirus, miembro de los *Reoviridae*, es uno de los principales causantes de gastroenteritis a nivel mundial. En el tercer mundo sus efectos son devastadores, al menos medio millón de muertes al año.

A pesar de que la replicación de rotavirus se produce en el citoplasma, el genoma viral -11 segmentos de RNA de doble hebra (dsRNA)- se oculta de los sistemas de defensa celulares gracias a los viroplasmas.

Los viroplasmas son estructuras citoplásmicas electrodensas visibles en la célula infectada poco después de la entrada de la partícula viral (3 a 4 horas post infección). Su formación es esencial para la replicación y los virus que fracasan en formar estas estructuras no producen partículas virales infecciosas. Las proteínas que forman el viroplasma son dos proteínas no estructurales, NSP2 y NSP5.

NSP2 es una proteína básica de 35 kDa, cuya estructura ha sido resuelta a través de cristalografía de rayos X. NSP2 ha sido caracterizada como una proteína desestabilizadora de doble hélice de RNA y también como una NTPasa y RTPasa.

NSP5 es codificada por el menor segmento de rotavirus, el gen 11. Esta es una proteína dimérica de 198 aminoácidos, ligeramente ácida, con una secuencia rica en serinas y treoninas.

NSP5 posee una afinidad por RNA de simple hebra (ssRNA) y de doble hebra (dsRNA). También, NSP5 ha sido caracterizada como una proteína que sufre modificaciones post traduccionales, especialmente ha sido acentuada la fosforilación e hiperfosforilación de ella *in vivo*. NSP5 es hiperfosforilada únicamente en presencia de

NSP2 *in vivo* tanto durante una infección como en el caso de una co-transfección de NSP2 y NSP5.

*In vitro* NSP5 posee una actividad autoquinasa que se ve incrementada en presencia de NSP2. NSP5 es fosforilada en mayor grado *in vitro*, pero esta fosforilación difiere de la hiperfosforilación *in vivo* que causa un cambio en el patrón de migración electroforética de la proteína. La actividad NTPasa de NSP2 afecta el grado de fosforilación de NSP5 *in vitro*, pero *in vivo* no se detectó ningún efecto de mutaciones en la actividad NTPasa de NSP2 sobre la fosforilación de NSP5 o la formación de viroplasmas.

Se ha identificado una nueva actividad enzimática de NSP5, una actividad ATPasa. Una caracterización de las condiciones de reacción de la hidrólisis *in vitro* permitió la determinación de las condiciones más convenientes para los ensayos de hidrólisis. NSP5 purificada de bacteria hidrolizó el enlace fosfodiéster β-γ del nucleótido. La hidrólisis sólo se llevó a cabo en presencia del catión divalente, $Mg^{+2}$. Otros nucleótidos no fueron hidrolizados por NSP5 con similar eficiencia en comparación con ATP.

No se ha detectado un efecto de RNA de simple hebra (ssRNA) de secuencia aleatoria o específica sobre la hidrólisis de ATP efectuada por NSP5. En el caso de RNA de doble hebra (dsRNA), este aumentó levemente la actividad ATPasa de NSP5.

En contraste a la autofosforilación de NSP5 *in vitro*, que es inducida por NSP2, la actividad ATPasa de NSP5 no fue afectada ni por la presencia de NSP2 ni por su actividad NTPasa.

Técnicas de simulación molecular permitieron postular un modelo de la estructura de NSP5 y de los aminoácidos sugeridos como críticos para la unión de ATP y de $Mg^{+2}$. Varios de estos aminoácidos fueron modificados a través de mutaciones sitio dirigidas y no se detectó un efecto significativo de estas mutaciones sobre la actividad ATPasa de NSP5 o la fosforilación de NSP5 *in vitro*.

Recientes reportes han identificado un residuo, S67, de NSP5 que ha sido propuesto como fosforilado por una quinasa celular, CKI, en la presencia de NSP2. De acuerdo con este modelo esta fosforilación sería esencial para la hiperfosforilación de NSP5 por otras quinasas celulares. A pesar de lo propuesto por el modelo, en el cual la serina 67 cumple un papel esencial en el proceso de fosforilación e hiperfosforilación de NSP5 *in vivo*, la mutación sitio dirigida del aminoácido 67 no causó mayor efecto tanto sobre la fosforilación de la proteínas *in vitro* como sobre la actividad ATPasa de NSP5.

Por lo tanto, varias evidencias sugieren que la fosforilación e hiperfosforilación de NSP5 *in vivo* no estarían relacionadas con su actividad ATPasa o su fosforilación *in vitro*.

Secuencias de NSP2 y NSP5 obtenidas de cepas de laboratorio de diferentes genotipos y de muestras provenientes de pacientes fueron analizadas. Estas son altamente conservadas, especialmente las secuencias de NSP5. Las secuencias aminoácidicas de NSP2 y NSP5 mostraron una distribución similar de las distancias entre cepas a partir de un análisis filogenético, pero no se encontraron correlaciones directas entre los cambios en la secuencia de NSP2 en relación a la secuencia de NSP5. Se detectó también una correlación interesante entre el origen de la secuencia

(proveniente de cepas de laboratorio o muestras de pacientes) y la manutención del segundo marco de lectura del gen 11, el marco de lectura de NSP6.

*"At the basis of the whole modern view of the world lies the illusion that the so-called laws of nature are the explanations of natural phenomena."(Wittgenstein, 1922)*

# INTRODUCTION

## 1. History and Epidemiology

Rotaviruses were first detected using electron microscopy in 1973 (Bishop *et al.*, 1973), and identified as the main cause of viral gastroenteritis in infants under the age of five. While the main concern regarding this virus in first world countries is with the high costs of hospitalization and treatment, the virus has even more devastating effect in third world countries, where 1 in every 300 children under the age of 5 will die as a result of gastroenteritis caused by rotaviruses (more than 500,000 deaths each year worldwide) (Parashar *et al.*, 2003).

Classification of rotavirus serotypes is based on two independent neutralization antigens, the proteins from the outer capsid VP7 and VP4 which are classified in G types (glycoprotein) and P types (protease sensitive) respectively (Hoshino and Kapikian, 1996).

Major advances in the development of vaccines against rotaviruses have been made. During the last decade, several versions of attenuated vaccines have been tried, although a concern was raised when a correlation between rotavirus vaccines and intussusceptions was detected and thus the vaccine use was paused. Today, major studies are analyzing the possible relation between vaccination and intussusception in

children as well as strategies to prevent this effect (Simonsen *et al.*, 2005). Several countries are already vaccinating against the virus, Brazil, Mexico and soon India and China. Thus, reports regarding large scale data on the effective consequence of the vaccine against different serotypes will be available soon and analyzed (Dr. A Kapikian, personal communication).

## 2. Virus Structure and structural proteins (VPs)

Rotavirus infective particle has an icosahedron structure composed of three concentric protein layers which entrap inside eleven segments of double stranded RNA (dsRNA), the genome of the virus (Estes, 2001; Prasad *et al.*, 1996). Recent cryo-electron microscopy (cryo-EM) studies have detected the compression and decompression of the genome inside the inner layer of the virion, the core, when exposed to different conditions (ionic strength, pH etc.), suggesting the isomeric and concentric organization of the condensed genome is due to strong interactions between the core proteins and the RNA (Pesavento *et al.*, 2001).

The outer protein layer consists of VP7 and VP4 (figure 1), a glycoprotein and a spike protein respectively (Estes, 2001). VP7 is the major backbone of this protein layer, made with 780 copies of the peptide, forming a T=13 symmetry (Estes, 2001; Prasad *et al.*, 1988). A hundred and twenty copies of VP4, a dimeric protein, are arranged forming spikes that extrude from the VP7 matrix (Prasad *et al.*, 1988).

The intermediate layer is composed of VP6, the most abundant rotavirus protein upon infection, 260 trimers also organized in a T=13 symmetry (Prasad *et al.*, 1988).

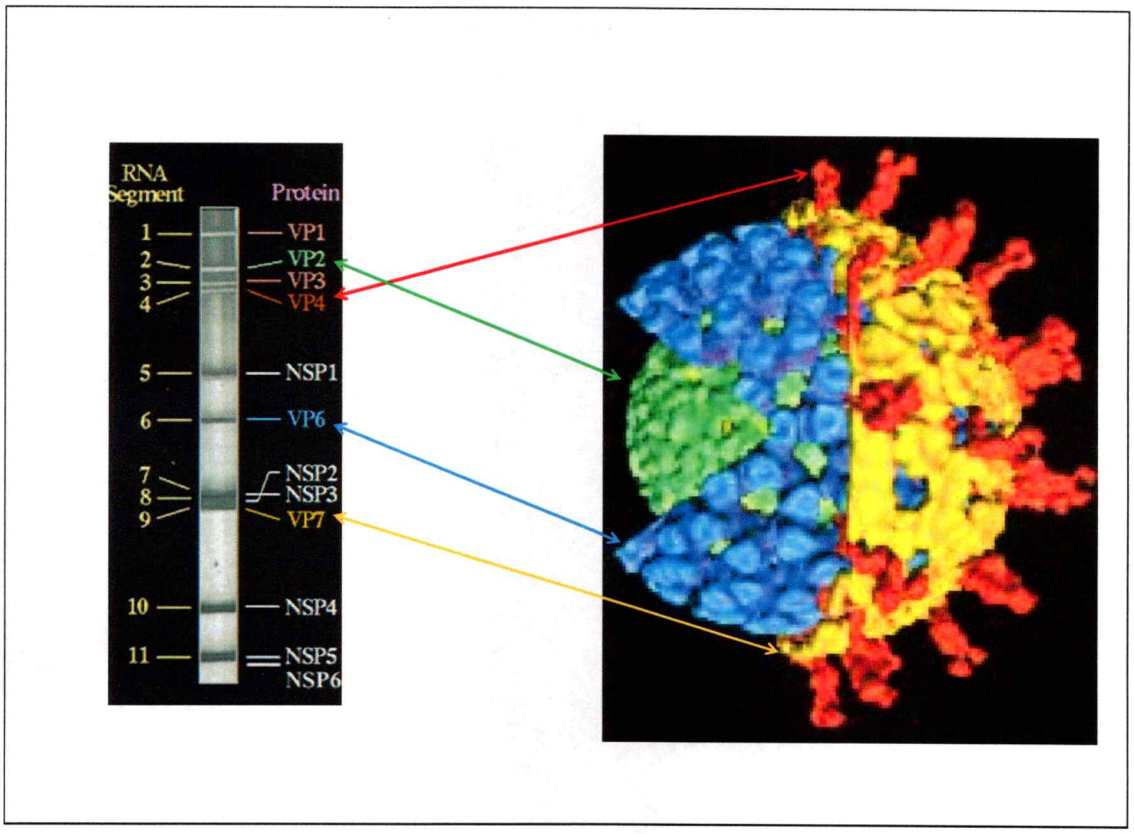



**Figure 1: Virion structure and composition.** On the *Left* a polyacrylamide gel showing the 11 segments of dsRNA from strain SA11 that constitutes the viral genome. The genomic segments are enumerated on the left and the proteins they codify are specified on the right in colors. On the *Right* a representation of the three dimensional structure of the virus particle. Connected with arrows, the structural proteins illustrated - VP7 and VP4 in the external layer, VP6 in the middle layer and VP2 in the core (adapted from (Estes, 2001).

The inner most layer, the core, has a T=1 symmetrical structure mainly composed of VP2 (Gallegos and Patton, 1989; Lawton *et al.*, 1997a; Lawton *et al.*, 1997b; Prasad *et al.*, 1988). VP2 is fastened to pentamers formed of 5 dimers, which in turn form 12 vertices (Prasad *et al.*, 1988). It is believed the core has a copy of VP1 and VP3 in most of its vertices (Lawton *et al.*, 1997b; Prasad *et al.*, 1988).

VP1 is the viral RNA dependent RNA polymerase of rotaviruses that synthesizes the (+)RNA transcripts while VP3 is believed to be responsible for the methyltransferase and guanylyltransferase activities, thus capping the nascent transcript (Estes, 2001; Liu *et al.*, 1992; Pizarro *et al.*, 1991; Sandino *et al.*, 1986).

Several structural channels, either on the vertices or near them, have been detected on DLPs, (double layer particles composed of the core and the VP6 intermediate layer). The channels have been classified according to their location or characteristics into three groups, type I, II and III. Since, as specified later, DLPs are transcription machineries during the viral cycle, it is believed the role of the channels is the extruding of the nascent mRNA out of the core and allowing the entering of nucleotides and other metabolites necessary for transcription (Estes, 2001; Lawton *et al.*, 1997a; Sandino *et al.*, 1986).

### 3. Non structural proteins

Rotavirus encodes for 6 structural proteins and 6 non structural proteins (figure 1) (NSPs) (Estes, 2001). NSP2, NSP5 and NSP6 will be discussed in detail later. Briefly, NSP2 and NSP5 are essential for viroplasm formation and the function of NSP6 is unknown.

An important topic studied in detail is the strategies of the virus to evade the innate immunity defenses of the cell. One of the pathways for such evasion is the interaction of NSP1 with the transcription factor IRF-3. This transcription factor is responsible for the transcription of INF-α, an interferon synthesized as a response to viral infection. NSP1 has been reported previously as a viral protein with high sequence variation and as a non essential protein for the viral cycle since viruses with silencing mutations, non-expressing NSP1 or expressing a truncated NSP1, were able to replicate in cell culture. Further studies indicated the replication of a virus without an active NSP1 was less efficient in cell culture and is probably selected against in infected animals (Barro and Patton, 2005; Graff *et al.*, 2002; Kearney *et al.*, 2004). Today several analyses are being made to check a possible co-evolution between NSP1 and IRF-3 from different strains and animals (Dr. M Barro and Dr. J Patton- personal communication). It seems NSP1 also alters other cellular pathways in order to evade the cell response to infection (Dr. M Barro and Dr. J Patton- personal communication).

NSP3 has a role in viral protein translation. The nascent (+)RNAs of rotaviruses are capped and lack a polyadenylated tail. During viral translation, NSP3 recognizes specifically the last nucleotides of the viral mRNAs and binds to the cellular factor eIFG4 that recognizes cap structures. In group A of rotaviruses the sequence in the 3' UTR recognized by NSP3 is 5'-UGUGACC-3'. Such an interaction between NSP3 and eIFG4 allows the circularization of the mRNA and an efficient protein synthesis (Piron *et al.*, 1998; Poncet *et al.*, 1994; Vende *et al.*, 2000).

Recent studies with siRNA for silencing of NSP4 have raised more questions regarding the roles of NSP4 in the replication cycle (Lopez *et al.*, 2005a; Silvestri *et al.*,

2005). NSP4 is believed to be a viral toxin since it interacts with $Cl^-$ channels in the epithelium and thus alters the fluid traffic across the intestine (Estes, 2001). Silencing of NSP4 in infected cell monolayes has shown a phenotype of accumulation of cores and non formation of infective virions (Silvestri *et al.*, 2005). Also, VP6 accumulates under such conditions in the cytoplasm. These phenomena could be related to the interaction of VP6 to NSP4, important for the internalization of DLPs into the ER (Lopez *et al.*, 2005a; Silvestri *et al.*, 2005).

## 4. Viral Infection and Replication cycle

Studies utilizing animal models show rotaviruses infect epithelium cells causing morphologic changes in the upper small intestine and causing diarrhea (Kapikian *et al.*, 2001). The replication cycle of rotaviruses has been mainly studied in cell culture using monkey rotavirus strains in monkey cell lines (specifically kidney monkey cells - MA104). The viral cycle is believed to be completely cytoplasmic (Estes, 2001).

Since rotaviruses produce very efficiently a high number of infective particles, many questions have been raised regarding the process of selection of the 11 segments of RNA, which ought to be precise in order to form an infectious particle (Patton and Spencer, 2000). Thus, although yet not fully understood, during the encapsidation and virus assembly the virus recognizes 11 different segments of ssRNA and encapsidates them in parallel to their replication. It is believed both, the sequence and the structure of the ssRNA segments have a role in this recognition process (Patton and Spencer, 2000; Tortorici *et al.*, 2006). The pour understanding of the assortment and encapsidation processes is also attributed the lack of a reverse genetic system, discussed later.

The steps of adsorption and penetration of the virus to the cell as well as its uncoating are not fully understood. It is believed rotaviruses enter the cell neither by caveolar endocytosis nor by clathrin uptake dependent manner, although cholesterol and dynamin take part of the internalization process (Sanchez-San Martin *et al.*, 2004). It has been suggested that after an initial contact between the particle and the cell surface through VP5 or VP8 or both (VP5 and VP8 are the result of the trypsin-treated VP4), VP4 undergoes a conformational change which allows VP4 and VP7 to contact other cell surface molecules. VP7 for example has been reported to interact with αvβ3 integrin although it hasn't been determined whether this interaction is related to the internalization process directly (Guerrero *et al.*, 2000a; Guerrero *et al.*, 2000b; Sanchez-San Martin *et al.*, 2004; Zarate *et al.*, 2004).

Upon entry of the particle to the cytoplasm the outer layer is lost and the particles become double layer particles (DLPs) (Patton, 1990). The DLP has been characterized as a transcription machinery, which transcribes 11 (+)RNAs, capped and non-polyadenylated (figure 2). The transcription is believed to begin immediately after viral penetration and its products, mRNAs, can be detected as soon as an hour after infection (Patton, 1990). Transcription products might be used either as templates for translation of viral proteins, with the participation of NSP3, or as templates for (-)strand synthesis (viral replication) rendered as dsRNA segments (Gallegos and Patton, 1989; Helmberger-Jones and Patton, 1986). Three dimensional visualization of active DLPs during transcription allowed to formulate a model of the extruding process of the nascent transcripts through channels of VP2 and VP6 (Lawton *et al.*, 1997a; Lawton *et al.*, 1997b).

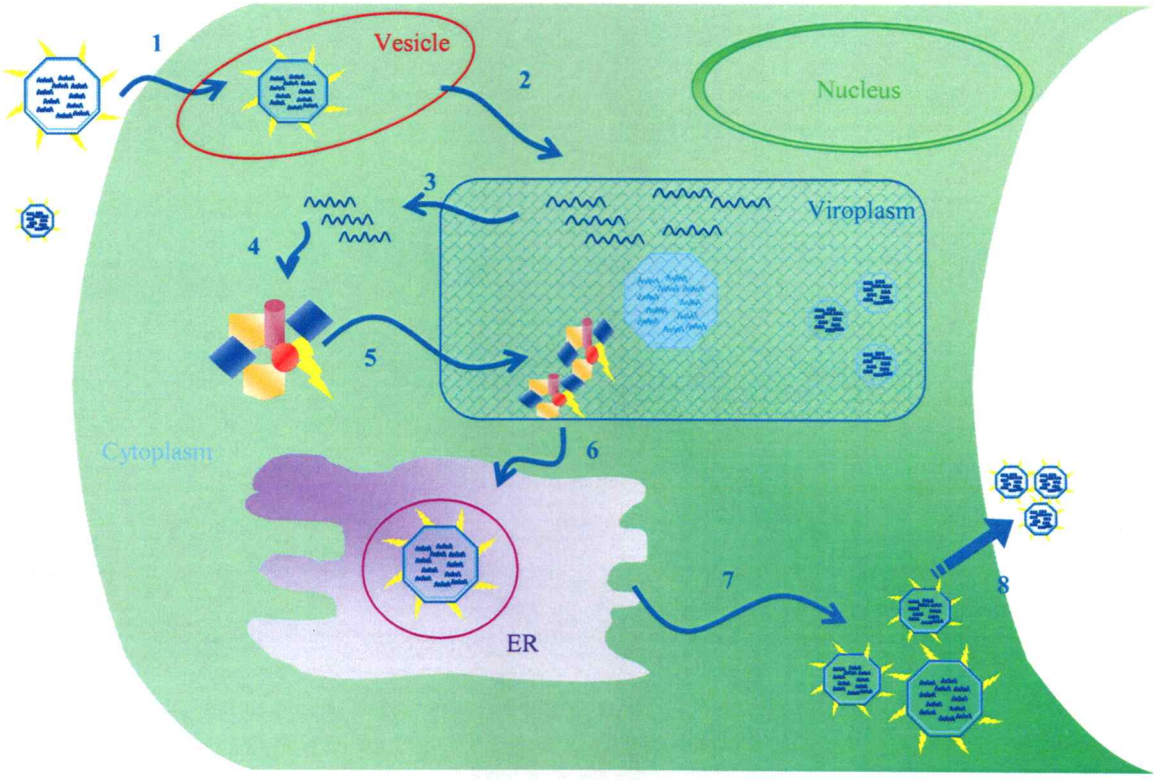



**Figure 2: Major features of the rotavirus infectious cycle.** 1) Adsorption and penetration to the cell. 2) Loose of the external protein layer to yield a DLP. 3) The synthesis of (+) RNAs is believed to take place in the viroplasms. Templates for translation are extruded from the viroplasm. 4) Translation of viral proteins assisted by NSP3. 5) Viral proteins are used in particle formation in the viroplasm. DLP particles can begin a new cycle of transcription inside the viroplasm or be captured by the ER (6) yielding an enveloped TLP intermediate. 7) TLPs loose the membrane as they exit the ER and following cell lysis 8) infectious virus particles exit the viroplasm.

The (-)RNA synthesis has been suggested to take place in electrodense perinuclear structures called viroplasms, formed by NSP2 and NSP5 (Estes, 2001). Intermediates of replication (RIs) formed by VP1, VP3, NSP2, NSP5 and (+)RNA have been detected. It is believed when VP2 joins this intermediates the negative strand begins to be synthesized while VP2 begins the formation of the pentameric vertices. VP2 has been shown to interact with NSP5 thus supporting an image of a continuous process from the formation of RIs to an interaction between the VP2 pentamers as the NSP2 and NSP5 are displaced, the core closes as the replication finishes and a layer of VP6 covers the cores forming DLPs (Berois *et al.*, 2003; Gallegos and Patton, 1989; Gonzalez *et al.*, 2000; Helmberger-Jones and Patton, 1986; Vende *et al.*, 2003).

Following its formation close to the periphery of the viroplasm, the DLP is directed to the ER (figure 2). It is believed this ER localization is the result of an interaction between NSP4 and VP6 (Estes, 2001).

In the process of internalization of the DLP to the ER the particle acquires its external layer, VP4 and VP7, as well as a lipid membrane which will be lost as the triple layer particle, TLP, leaves the ER. It is believed the exit of the TLP from the ER is due to a partial proteolysis of VP7 which affects the TLP interaction with NSP4 causing the loss of the membrane. TLPs then accumulate in the cytoplasm and cause cell lysis thus liberating the viral particles (Estes, 2001; Gonzalez *et al.*, 2000).

## 5. Viroplasms

The replication cycle of double stranded RNA viruses, including the family of *Reoviridae*, has to deal with a possible exposure of its genome, dsRNA, to the defense systems of the cell which might be activated. Rotaviruses replication takes place in the cytoplasm while the dsRNA is kept 'hidden' from the systems of defense of the cell thanks to the viroplasm (figure 3). Viroplasms are visible in the infected cells shortly following the entrance of the virus particle (3 to 4 hours post infection) (Patton and Spencer, 2000). Their formation is essential for replication and viruses that fail to form these structures, for example temperature sensitive strains in non permissive temperatures (e.g. *tsE-NSP2*), do not form infective particles (Taraporewala *et al.*, 2002).

The scaffold of the viroplasms is constituted of two non structural proteins, NSP2 and NSP5. Both proteins are able to form viroplasm like structures (VLS) in the absence of other viral proteins or viral RNA's when co-transfected in MA104 cells (Fabbretti *et al.*, 1999). Recently it has been reported that NSP5 alone, when tagged in its N-termini with GFP, is also able to form viroplasm like structures (Mohan *et al.*, 2004) thus suggesting that a feature of NSP2 in viroplasm formation is its binding to the N-termini of NSP5.

The transcription of viral mRNA gives rise to templates for two processes, viral replication and viral protein translation (figure 3). Regarding the destiny of the (+)RNA transcripts, Silvestri *et al.* (Silvestri *et al.*, 2004) showed that while the transcripts which are templates for the replication process are protected from siRNA-directed degradation, (+)RNAs which were templates of translation were not protected. That suggested the transcripts might be synthesized inside the viroplasm structures and then exported

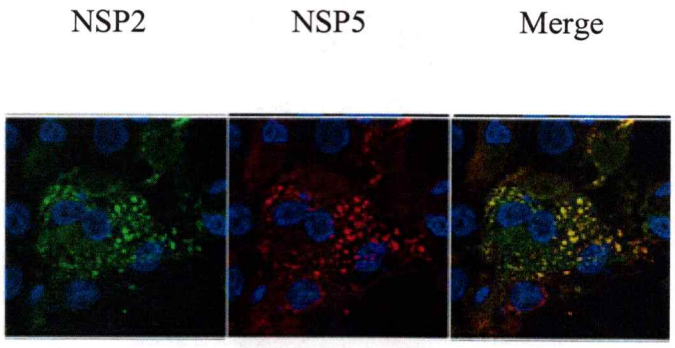
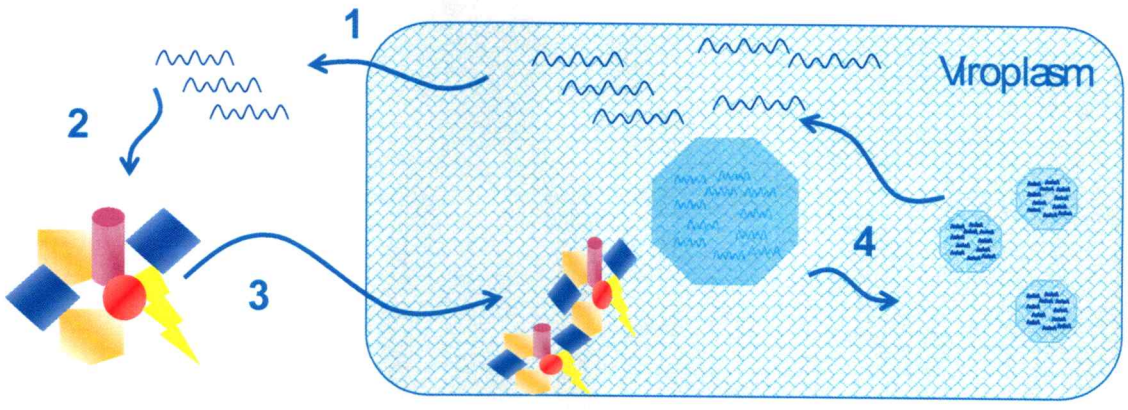



**Figure 3: Viroplasms and replicative cycle.** *Up* – MA104 cells co-transfected with NSP2 and NSP5. Immunofluorescence and confocal microscopy image. Blue- Nucleus stained with DAPI, red, NSP5, green, NSP2 and in yellow co-localization- VLPs. (Adapted from (Vasquez del Carpio *et al.*, 2004). *Down*- Model of replication cycle focusing in the viroplasm. According to the results by (Silvestri *et al.*, 2004) the (+)RNAs are protected from degradation by siRNAs, suggesting transcription takes place in the viroplasm (1), (+)RNAs are extruded from the viroplasm (2) and translated in the cytoplasm; (3) Movement of viral proteins from transcription sites to the viroplasm. Following DLP formation DLPs may enter a new cycle of transcription (4) in the viroplasm.



outside the viroplasm to serve as templates for translation (Silvestri *et al.*, 2004). This might be also a reason for the non succeeded attempts to introduce a foreign segment into rotaviruses since the viroplasms protect the (+)RNAs to be used in the replication and foreign RNAs might not be able to be imported inside the viroplasm structure for a further replication and encapsidation.

### 6. NSP2

NSP2 is a basic protein of 35 kDa, encoded by gene 8, with several described activities, essential for replication. A temperature sensitive mutant of NPS2, *tsE*, is incapable of replication and formation of infective viral particles in non permissive temperatures (Taraporewala *et al.*, 2002). NSP2 forms stable 10S octamers in solution whose structure was characterized through crystallization and cryo-EM studies (Jayaram *et al.*, 2002; Schuck *et al.*, 2001). NSP2 has an affinity for ssRNA which has been defined as non specific. Analytical ultracentrifugations (AU) determined small changes in the structure of the octamer induced in the presence of $Mg^{+2}$ and ATP, as well as ssRNA. When compared to the *wt* protein, *tsE*-NSP2 showed a deficient octamer formation (Jayaram *et al.*, 2002; Schuck *et al.*, 2001; Taraporewala *et al.*, 1999; Taraporewala *et al.*, 2002).

*In vitro* the protein was phosphorylated when incubated with [$\gamma$-$^{32}$P]-ATP although *in vivo* it was not detected in a phosphorylated form (Taraporewala *et al.*, 1999; Vasquez del Carpio *et al.*, 2004). In following studies, it was shown that NSP2 has an NTPase activity hydrolyzing indiscriminately all four NTPs to the same extent (Taraporewala *et al.*, 1999). It is thought the phosphorylated form detected *in vitro* is an

intermediate of reaction during the hydrolysis of the NTP. Further analysis demonstrated that the NTPase activity is not directly involved in the formation of viroplasms since mutants in this enzymatic activity do not affect viroplasm formation (Taraporewala *et al.*, 1999; Vasquez del Carpio *et al.*, 2004).

A hypothesis regarding a possible helicase activity of NSP2 brought about from its particular doughnut structure and its ability to hydrolyze nucleotides was tested. NSP2 showed a dsRNA helix destabilizing activity maintaining the assumption of a possible role in the destabilization of secondary structures in RNA during replication or encapsidation (Taraporewala and Patton, 2001). In spite of this result NSP2 differs from described helicases since this destabilizing activity was independent from the presence of $Mg^{+2}$ and nucleotides (Schuck *et al.*, 2001; Taraporewala *et al.*, 2002).

Lately, NSP2 has been shown to hydrolyze the 5'-γ phosphate of ssRNA preferring this substrate to NTPs (Dr. J. Patton, Dr. Z. Taraporewala and Dr. R. Vasquez del Caprio- personal communication). This data suggests NSP2 might act as an RTPase *in vivo*.

### 7. NSP5 and NSP6

Gene 11, the smallest segment of rotaviruses, codes for two non structural proteins, NSP5 and the smallest NSP6 (Estes, 2001; Mohan and Atreya, 2001). Some viral strains have been reported to loose the second ORF of gene 11 without an apparent effect on the viral replication cycle. Thus, speculations about the expression and function of NSP6 rose (Estes, 2001; Mohan and Atreya, 2001).

NSP5 is a 26 kDa slightly acidic protein with a sequence extremely rich in serines and threonines. Up to date several characteristics of NSP5 are known. One of its main functions is its role in viroplasm formation and its co-localization with NSP2 in infected cells.

Another important characteristic is its ability to bind ssRNA and dsRNA, both in a sequence independent manner (Vende *et al.*, 2002).

A series of deletion mutations as well as yeast two hybrid studies showed several interactions between NSP5 and other viral proteins. NSP5 was proposed to interact with NSP2, NSP6 as well as dimerize. The region of the last 10 amino acids of NSP5 seems to be critical for the dimerization of the protein (Torres-Vega *et al.*, 2000).

When tagged in its N-termini with GFP, NSP5 forms viroplasm like structures which are not seen when NSP5 is tagged in its C-termini or not tagged (in the absence of NSP2), suggesting NSP5 interacts with NSP2 inducing the formation of viroplasm. The region of the N-termini of NSP5 seems to be important for such interaction (Mohan *et al.*, 2004; Sen *et al.*, 2006).

Some of the best known characteristics of NSP5 are its post-translational modifications. When purified from infected cells, NSP5 migrated at a molecular weight corresponding to 28kDa and 30-32 kDa, higher than its theoretical weight. Through chemical analysis it was determined by Gonzalez *et al.* in 1991 that NSP5 undergoes a NAc-O-Glycosylation (Gonzalez and Burrone, 1991). These modifications take place on serine or threonine residues in the cytoplasm. The role of this modification in this system is still unknown.

Later studies regarding the shift in molecular weight of NSP5 showed that it is correlated with the phosphorylation and hyperphosphorylation of the protein. This modification was able to shift the protein to migration at 28 kDa when phosphorylated and 30-32 kDa when hyperphosphorylated. NSP5 only undergoes hyperphosphorylation in infected cells or when co-transfected with NSP2 but not when it is transfected alone. Deletion studies of NSP5 in transfected cells identified regions suggested as critical for the phosphorylation and hyperphosphorylation of NSP5 in the absence of NSP2 in transfected cells. A characterization of the amino acids phosphorylated *in vivo* determined the amino acids phosphorylated *in vivo* are serines (Afrikanova *et al.*, 1996; Blackhall *et al.*, 1998; Eichwald *et al.*, 2002; Poncet *et al.*, 1997).

*In vitro* NSP5 was showed to undergo autophosphorylation in the absence of other viral proteins when incubated with [$\gamma$-$^{32}$P]-ATP. This phosphorylation was positively affected when NSP2 was present in the reaction *in vitro*. Thus, a hypothesis conceived was that NSP5 had an autokinase activity which is either further activated thanks to an induction of NSP2 or that the subsequent phosphorylation was due to an activity of NSP2 (Afrikanova *et al.*, 1998; Blackhall *et al.*, 1997; Poncet *et al.*, 1997). When NSP2 mutants in their NTPase activity were incubated with NSP5 *in vitro* the induction of the phosphorylation of NSP5 was abolished but these mutations had no effect on the phosphorylations and hyperphosphorylations of NSP5 *in vivo* in co-transfected cells (Vasquez del Carpio *et al.*, 2004). Thus, the NTPase activity of NSP2 has an effect on the phosphorylation of NSP5 *in vitro* but no apparent effect was seen *in vivo*.

A mutation of S67 of NSP5 was reported to have an effect on the phosphorylation and hyperphosphorylation of the protein *in vivo*. A proposed model suggested this is the first amino acid to be phosphorylated and after this phosphorylation NSP5 undergoes a structural change that exposes other sites for a posterior hyperphosphorylation. According to this model S67 was phosphorylated by a cellular kinase, CKI, while the serines phosphorylated afterwards were phosphorylated by CKII ((Eichwald *et al.*, 2002; Eichwald *et al.*, 2004) . This model has been questioned by a recent publication that argues *in vivo* the mutation of serine 67 has no effect on the states of phosphorylation and hyperphosphorylation of NSP5 (Sen *et al.*, 2006). In this work the importance of S67 was analyzed for the activities of the protein *in vitro*.

One of the most important tools in the study of the function of non structural proteins is an analysis of its structure. The structure of NSP5 remains unsolved. Also, the identification of the amino acids phosphorylated *in vivo* is difficult since the sequence of NSP5 is constituted of more than 30% serines and threonines.

Silencing experiments confirmed recently NSP5 has a critical role in the replication cycle of the virus. When targeted with siRNA or with intrabodies, an infected cell was unable to form viroplasms and as expected no infective particles were synthesized (Campagna *et al.*, 2005; Lopez *et al.*, 2005b; Vascotto *et al.*, 2004).

The relation between the posttranslational modifications of NSP5, in particular its phosphorylation and hyperphosphorylation, and the protein functions were not proven yet. Only in one report a connection between the amounts of protein synthesized, viral genomic RNA and the phosphorylation of a mutant of NSP5 has been suggested (Chnaiderman *et al.*, 2002). It is still unclear which functions of NSP5 are affected by its

level of phosphorylation as well as whether phosphorylation and hyperphosphorylation are related to critical functions like viroplasm formation.

Comparisons are made between proteins with similar characteristics in the *Reoviridae* trying to find relationships regarding functions or other properties of another. Several viral proteins have been compared to NSP5 and NSP2, some also being critical for cytoplasmic inclusion formation and thus virus replication. Questions were raised regarding the parallel functions and characteristics of these proteins. For example, NS2 of Blue Tongue Virus (BTV), has been hypothesized as a 'hybrid' between NSP2 and NSP5 due to its common properties with both (Horscroft and Roy, 2000; Modrof *et al.*, 2005; Taraporewala *et al.*, 2001).

During an analysis of the *in vitro* autophosphorylation activity of NSP5, in order to characterize this activity, we tested a new hypothesis- does NSP5 has other enzymatic activities? Does rotavirus, like reovirus, have more than one viral NTPase or RTPase? Is NSP5, similarly to other viral proteins, related to cytoplasmic structure formation necessary for replication cycle, has an NTPase activity?

When checked for the ability to hydrolyze NTPs, NSP5 showed an ATPase activity hydrolyzing the β-γ phosphodiester bond of ATP.

Thus, following the discovery of a new enzymatic activity of NSP5, several questions regarding this activity were raised. Is NSP5 an NTPase without any specificity for the base of the nucleotide to be hydrolyzed? Is this activity affected by the presence of NSP2? Is the NTP hydrolysis of NSP5 more effective than the hydrolysis of NSP2?

## HYPOTHESIS AND OBJECTIVES

Following these arguments a hypothesis and several objectives were raised:

### Hypothesis

NSP5 is a phosphoprotein with an ATPase activity.

### General objective

Characterize the ATPase activity of NSP5 *in vitro*.

### Specific Objectives

1. Characterize the ATPase activity of NSP5.
2. Analyze the effect of the presence of NSP2 and RNA on the ATPase activity of NSP5.
3. Determine the relation between the phosphorylation and ATPase activity of NSP5.

## MATERIALS AND METHODS

### 1. Expression Vectors and site directed mutagenesis

The bacterial expression vectors pQE60g8 and pQE30g11 encode for NSP2 with a 6-His C-terminal Tag and NSP5 with a 6-His N-terminal tag respectively. The NSP2 mutants analyzed were described before (Vasquez del Carpio *et al.*, 2004). pQE60g11 was mutated by PCR using Platinium *Pfx* DNA polymerase (Invitrogen). The primers used for the mutagenesis are listed in table 1.

Following amplification (94°C for 2 min, 1 cycle; 94 °C for 15 sec, 47 °C for 30 sec, 68 °C for 5 min 30 sec, 10 cycles; 94 °C for 15 sec, 52 °C for 30 sec, 68 °C for 5 min 30 sec, 25 cycles; 68 °C for 10 min, 1 cycle) the PCR products were gel purified, self ligated using a Quick Ligation kit (New England Biolabs) and transformed into DH5α (Invitrogen). The mutations were verified by sequencing and the plasmids were transformed into M15 [Rep4].

### 2. Expression and purification of NSP5, NSP2 and mutants

M15 [pREP4] bacteria containing pQE60-NSP2 *wt* or mutants or containing pQE30-NSP5 *wt* or mutants were grown in Terrific Broth (Quality Biologics) at 37°C to an $OD_{600}$ of 0.5. Expression was induced adding IPTG (Sigma) to a final concentration of 1 mM. Four to five hours after induction the bacteria were recovered by centrifugation at 4000 *g* for 30 min. The proteins were purified from bacterial lysates under native conditions on Ni-NTA (Nickel-Nitrilotriacetic acid) agarose columns according to the manufacturer protocol (Qiagen) and as previously reported (Vasquez

del Carpio *et al.*, 2004; Vende *et al.*, 2002). Then, NSP2 *wt* was dialyzed overnight against low salt buffer (LSB) (2 mM Tris-HCl pH 7.5, 0.5 mM EDTA and 0.5 mM dithiothreitol) and NSP2 mutants were dialyzed against LSB with a final NaCl concentration of 25 mM. NSP5 *wt* and mutants were shortly dialyzed against 50 mM $NaH_2PO_4$ pH 8, 300 mM NaCl and extensively dialyzed in LSB with 75 mM NaCl and 0.1% Triton X-100. Protein concentrations were determined by Bradford assay using bovine serum albumin (BSA) as the standard and by co-electrophoresis with known amounts of BSA by 14% SDS-PAGE (Novex Tris-Glycine-Invitrogen) followed by Coomassie Blue staining.

### 3. Western blot analysis

Proteins were separated in 14% SDS-PAGE (Novex Tris-Glycine-Invitrogen) and transferred onto a nitrocellulose membrane (0.45 μm pore size-Invitrogen). The blot was probed sequentially either with 1:10,000 guinea-pig anti NSP5 polyclonal antisera and goat anti-guinea pig horseradish peroxidase-conjugated antibody (1:10,000) or with 1:5,000 mouse monoclonal penta-His antibody (Qiagen) and a goat anti mouse antibody (1:5,000).

### 4. NTPase assay

Overall, reaction mixtures for the NTPase assay contained 2 μg of NSP5, 50 mM Tris-HCl (pH 7.5), 200 μM $MgCl_2$, 150 mM NaCl and 10μCi of [$\alpha$-$^{32}$P] ATP (800 Ci/mmol, Perkin Elmer) in a final volume of 15μl. The mixtures were incubated at 37°C

for 1 hour and stopped with 10 mM EDTA. Following phenol-chloroform extraction 1 µl of reaction mixture was spotted on polyethyleneimine-cellulose thin layer chromatography (TLC) plate and was developed in 1.2 M LiCl. The reaction products were visualized and quantified using a Phosphoimager. The percentage of hydrolysis of ATP was calculated using the following formula: ATP hydrolysis = ((quantity of [α or $\gamma$-$^{32}$P] ADP or Pi) / (quantities of [α or $\gamma$-$^{32}$P] ADP or Pi + [α or $\gamma$-$^{32}$P] ATP)) x 100. In each experiment the hydrolysis was corrected subtracting the value obtained in a reaction mixture without protein.

The protein DnaK and the antibody against DnaK used in western blot assays and ATP hydrolysis assays were obtained from Stressgen Bioreagents.

**5. Photocrosslinking assays**

Reaction mixtures for photocrosslinking assays typically contained 2 µg *wt* or mutant NSP5, 50 mM Tris-HCl (pH 7.5), 200 µM $MgCl_2$, 150 mM NaCl and 0.1 µCi of 8-azido-[α-$^{32}$P] ATP (20 Ci/mmol) (Affinity Labeling Technology). Following incubation for 5 minutes on ice the reaction mixtures were exposed for 1 and a half minutes under a lamp of 254 nm UV light located at 4 cm from the sample. When crosslinked to the protein, a covalent bond is formed between the azido group activated by the UV light and a nearby protein residue, allowing an analysis through 14% SDS-PAGE (Novex Tris-Glycine-Invitrogen) and posterior autoradiography detection and Phosphoimager quantification.

### 6. Competition assay

Reaction mixtures for the NTPase competition assay contained 2 μg of NSP5, 50 mM Tris-HCl (pH 7.5), 200 μM $MgCl_2$, 150 mM NaCl and 10μCi of [α-$^{32}$P] ATP (3,000 Ci/mmol, Perkin Elmer) and cold nucleotides (cold ATP (Invitrogen), cold GTP (Ambion) or cold UTP (Ambion)) in the indicated concentration in a final volume of 15μl. The mixtures were incubated at 37°C for 1 hour. ADP and ATP mixtures were resolved by an ascending TLC in 1.2 M LiCl.

### 7. *In vitro* synthesis of ssRNAs and dsRNAs

Sequence specific ssRNA, g8 5' 21 mer was obtained by an *in vitro* transcription kit (Ambion) as previously described (Vasquez del Carpio *et al.*, 2004). Poly U was obtained from Sigma. Double stranded RNA is a sequence specific 22 mer siRNA (SA11g2-1345si) (Invitrogen).

### 8. Autophosphorylation of NSP5

Reaction mixtures for NSP5 autophosphorylation analysis contained 2 μg of *wt* or mutant, 50 mM Tris-HCl (pH 7.5), 200 μM $MgCl_2$, 150 mM NaCl and 10μCi of [γ-$^{32}$P] ATP (3,000 Ci/mmol, Perkin Elmer) in a final volume of 20 μl and were incubated for 2 hours at 37°C. Phosphorylated proteins were separated by 14% SDS-PAGE (Novex Tris-Glycine-Invitrogen), detected through autoradiography and quantified with a Phosphoimager.

## 9. Hydrolytic Stability of the phosphoamino acid in NSP5 under Acid or Alkaline Conditions

Reaction mixtures for NSP5 and NSP2 autophosphorylation contained 2 μg of NSP5 or 1 μg of NSP2, 50 mM Tris-HCl (pH 7.5), 200 μM $MgCl_2$, 150 mM NaCl and 10μCi of [$\gamma$-$^{32}$P] ATP (3,000 Ci/mmol, Perkin Elmer) in a final volume of 20 μl and were incubated for 2 hours at 37°C. Phosphorylated proteins were separated by 14% SDS-PAGE (Novex Tris-Glycine-Invitrogen) and transferred to polyvinylidene difluoride membrane - PVDF (Invitrogen). Membranes were air-dried and autoradiographed directly or after an individual incubation at 65 °C for 2 hours in alkaline (1 M KOH) or acid (6 M HCl) conditions.

## 10. Molecular dynamics - Model of NSP5

The sequence of SA11 NSP5 was analyzed in the PDB (GCG) database. A protein with a similarity higher than 30% with NSP5 sequence was found- a cytoplasmic ribonuclease- with the PDB access number 1B6VB. This similarity allowed the construction of a model of NSP5 through homology. Following the construction of the model with the program *MODELLER*, a relaxation using the program *CHARMm28* was performed. In order to keep secondary structures during the relaxation process energetic restrictions of the peptidic angles were set. After the relaxation of the model, a search for the binding site of ATP, docking, was done with the program *AUTODOCK 3.02*.

Based on the results of this preliminary model a putative binding site of ATP was detected and three amino acids were identified as possible targets that could affect the

ATP binding. These amino acids, Arg 144, Lys 145 and Arg 186, were mutated through site directed mutagenesis.

Also, determination of putative amino acids crucial for $Mg^{+2}$ binding was identified following results indicating that this cation was essential for the ATPase activity. Based on this model the putative amino acids critical for the cation binding were glutamic acid 151 and aspartic acid 153 which were also mutated through site directed mutagenesis.

**11. Sequences of NSP5 from field samples of infected patients and laboratory strains.**

Samples of Rotaviruses from field samples of infected patients and laboratory strains were gently contributed by Dr. Norma Santos (Universidade Federal do Rio de Janeiro) and Dr. Yasutaka Hoshino (LID/NIAID/NIH) respectively.

The alignments and phylogenetic analysis was done utilizing the bioinformatic tool *ClustalW* (http://www.ebi.ac.uk/clustalw/ ) version 1.82 with the default parameters ( DNA Gap Open Penalty = 15.0, DNA Gap Extension Penalty = 6.66, DNA Matrix = Identity Protein Gap Open Penalty = 10.0, Protein Gap Extension Penalty = 0.2, Protein matrix = Gonnet Protein/DNA ENDGAP = -1, Protein/DNA GAPDIST = 4).

**Table 1- Primers used for site directed mutagenesis.**

| | |
|---|---|
| S67A-Rev | 5' GTGAGTGGATCGTT**TG**CAGCAG 3' |
| S67D –Rev | 5' GTGAGTGGATCGTT**GT**CAGCAG 3' |
| S67 Ph–For | 5' CAGTTTTTCGATTAGATCG 3' |
| R144A – For | 5' CATAAAAGT**GC**GAAGCACTACCC 3' |
| R144A – Rev | 5' GTTTTGTTTTGATTTCTCCTTTTTAG 3' |
| K145A – For | 5' CATAAAAGTAGG**GC**GCACTACCC 3' |
| R186A – For | 5' CGCATTAAGAATG**GC**AATGAAA 3' |
| R186A - Rev | 5' AAGTATTTCTTCTTATATTTACAG 3' |
| E151D153A-Rev | 5' GGTAGTGCTTCCTAC 3' |
| E151D153A-For | 5' CAAGAATTG**C**AGCAG**C**GTG 3' |

Bases in primers used to change codons in the open reading frame of NSP5 are in bold.

# RESULTS

## 1. Purification of NSP5

NSP5 and NSP2 were expressed in bacteria using an IPTG inducible expression system. Both proteins have attached a 6 histidine tag on the C-termini or N-termini respectively. Following purification, both proteins were dialyzed against buffers with low salt concentrations. NSP2 is highly stable and soluble; it was dialyzed against LSB (Low salt buffer) overnight. When dialyzed in the same way, NSP5 precipitates in the dialysis cassette. Thus, the dialysis process of NSP5 was modified: the purified protein was dialyzed against a buffer with a high salt content and the salt concentration was lowered in a stepwise manner to reach a final concentration of 75 mM NaCl and 0.1% Triton X-100. Following the dialysis, the proteins were quantified through the Bradford assay. Purified NSP2 and NSP5 can be appreciated in figure 4. The identity of the protein was verified through Western blot against the His tag and against NSP5. NSP5 was co-purified with a contaminant, DnaK, which was identified (Vende *et al.*, 2002) and is less than 1% of the total protein purified (discussed later).

## 2. NSP5 has an ATPase activity.

NTPase activity has been described as the hydrolysis of a nucleotide triphosphate to a nucleotide diphosphate and a free phosphate (NTP->NDP+Pi).

NSP2 was previously characterized as an NTPase. According to previous studies NSP5 is a phosphoprotein which undergoes several phosphorylations both *in vivo* and *in vitro*. When analyzed, NSP5 had an ATPase activity. The analysis of ATP hydrolysis

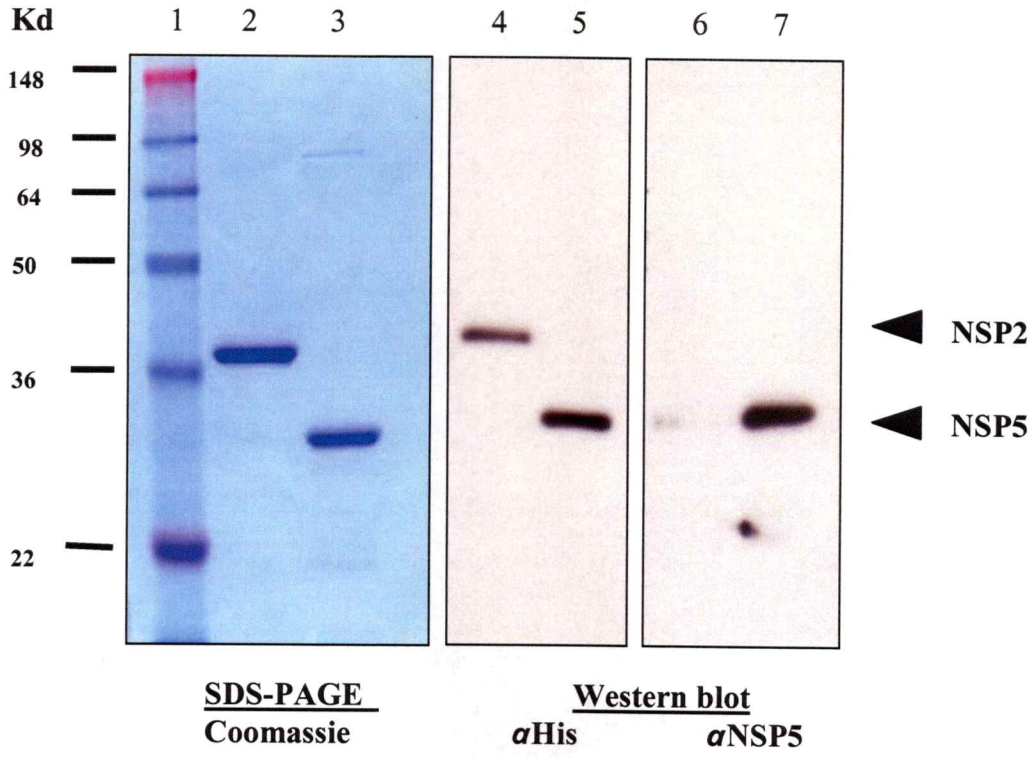
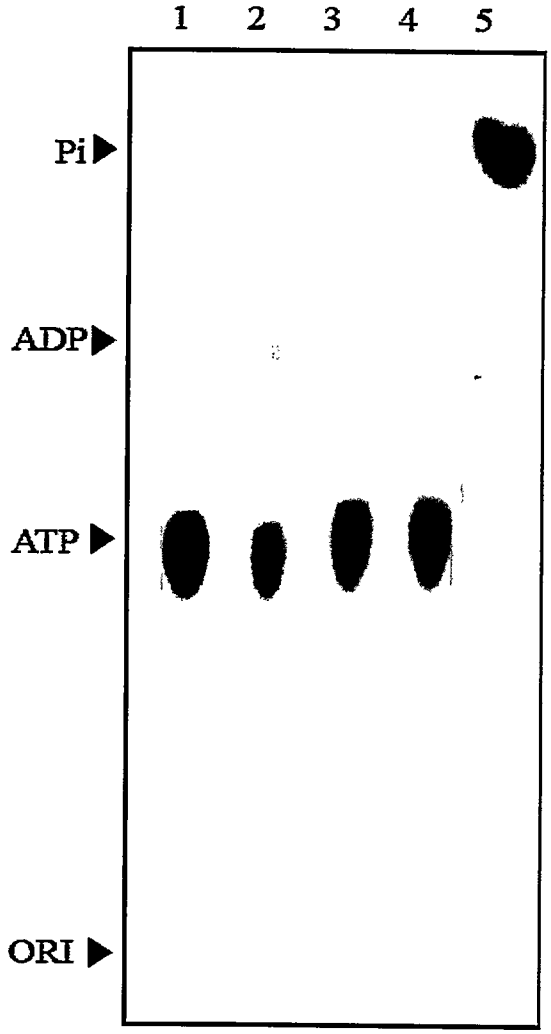



**Figure 4: Expression and purification of NSP5 and NSP2.** The proteins NSP5 and NSP2 were expressed in bacteria and purified through high affinity chromatography. Proteins were solved through an SDS-PAGE and Coomassie stained and also identified through western blot using anti-His antibody or anti-NSP5 antibody. Lanes: 1, protein molecular size standards; lanes 2, 4 and 6 His-tagged NSP2 eluted from Ni high affinity column; lane 3, 5 and 7 His-tagged NSP5 eluted from Ni high affinity column.

during this work was done using thin layer chromatography, autoradiography and further analysis and quantification using phosphoimager. NSP5 was able to hydrolyze more than 30% of the ATP in these conditions (figure 5). The hydrolysis seems to be $Mg^{+2}$ dependent since when EDTA (final concentration- 6.67 mM) was added, the ATP hydrolysis showed a critical decrease.

### 3. Comparison between the hydrolysis of ATP by DnaK and NSP5

As mentioned before, less than 1% of the protein purified through the Ni-agarose columns in NSP5 purifications is a contaminant identified as DnaK. DnaK is a heat shock protein in bacteria which possess also an ATPase activity. In order to rule out that the hydrolysis of ATP observed is the result of DnaK hydrolytic ability, commercially available DnaK was used. The hydrolysis of similar amounts of DnaK available commercially and a sample of NSP5 and its contaminant were tested under the same conditions for hydrolysis of ATP (figure 6). DnaK alone had much less hydrolytic activity than a sample of NSP5 with the contaminant. Also, when performing a hydrolysis assay at 55°C, NSP5 was highly affected while the hydrolysis by DnaK was not. Based on this assay as well as some other indications, for example the cation which is preferred in this reaction of hydrolysis, the conclusion was that the hydrolysis of ATP is mainly the result of the enzymatic activity of NSP5 and not the contaminant DnaK (Barthel *et al.*, 2001).




| | | | | | |
|---|---|---|---|---|---|
| NSP5 | - | + | + | + | - |
| T°C | 37 | 37 | 55 | 37 | 37 |
| EDTA | - | - | - | + | - |
| CIP | - | - | - | - | + |
| Hydrolysis (%) | 0 | 32 | 5 | <1 | 98 |

**Figure 5: ATPase activity of NSP5.** [$\alpha$-$^{32}$P] ATP hydrolysis in reaction mixtures, incubated for one hour at 37°C with no protein or with NSP5 and 10 μCi [$\alpha$-$^{32}$P] ATP, was detected by TLC and autoradiography. Lane 1: no protein added; lane 2-4 70 pmol of NSP5 in indicated conditions; lane 5: positive hydrolysis control- 10 units of CIP (Calf Intestine Phosphatase-New England Biolabs). Final concentrations: $Mg^{+2}$- 200 μM, ATP- 0.833 μM, NSP5 4.67 μM, NaCl 150 μM, EDTA 6.67 mM.

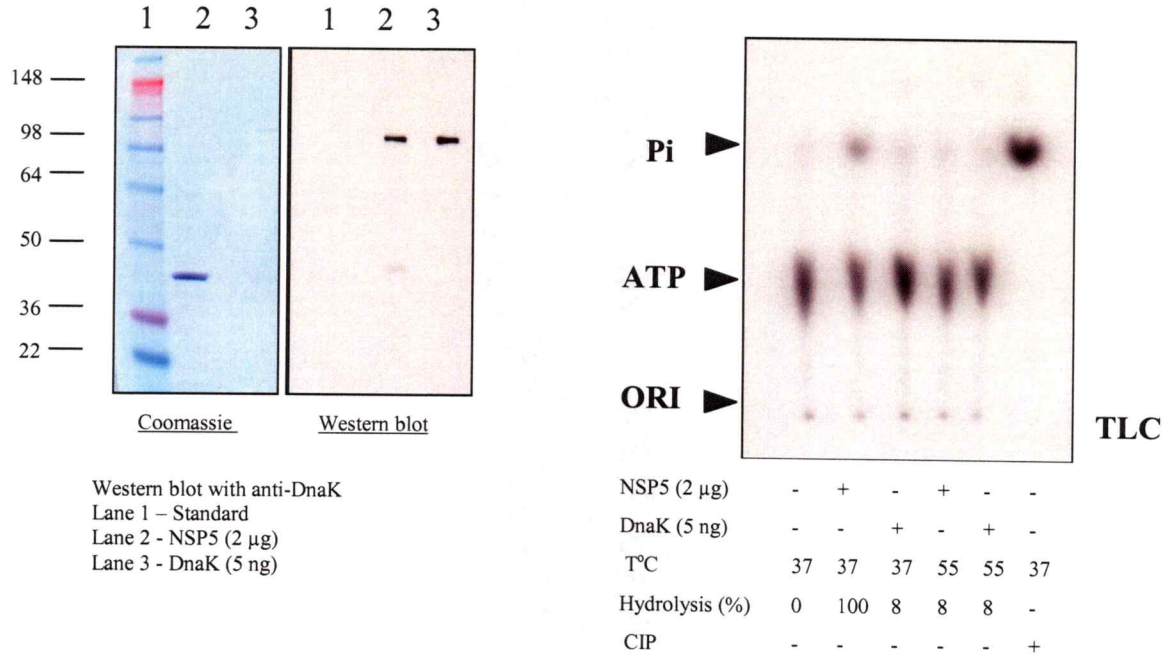



**Figure 6: Comparison between the hydrolysis of ATP by DnaK and NSP5.** *Left* - Proteins were solved through an SDS-PAGE and Coomassie stained and also identified through western blot using anti-DnaK antibody (Stressgen). With 2 µg of purified NSP5, around 5 ng of DnaK were identified (similar amounts seen in Coomassie and the Western blot) *Right*- TLC analyzing hydrolysis of [$\alpha$-$^{32}$P]-ATP in the conditions indicated. Five ng of DnaK were tested for ATP hydrolysis and compared to 2 µg of NSP5 co-purified with 5 ng of DnaK. Hydrolysis at 55°C was analyzed and compared as well. Most of the hydrolysis detected in an NSP5 sample (92%) is the result of the enzymatic activity of NSP5 and only a small portion is the result of the enzymatic activity of DnaK.

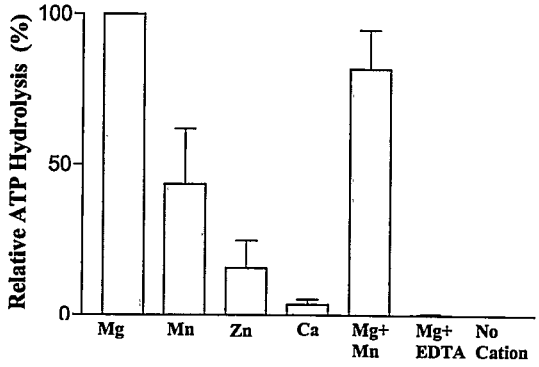
### 4. Characteristics of the ATPase activity

In order to make further analysis of the ATPase activity of NSP5 we optimized the reaction conditions.

The pH conditions were analyzed (figure 8A). Similarly to other proteins of rotavirus, NSP5 showed a higher enzymatic activity at a pH range of 7.5-8 (similar to the cytoplasmic cellular conditions).

When the different cations were analyzed we could clearly identify that NSP5 prefers $Mg^{+2}$ over $Mn^{+2}$ or any other divalent cation. There was no detection of a synergic effect when $Mg^{+2}$ and $Mn^{+2}$ were used together. Other cations did show only a slight induction of the hydrolysis of ATP. The hydrolysis was totally dependent of the presence of divalent cations (figure 7).

The ATPase activity may be influenced by the ionic strength conditions in the reaction mixtures. In this context two different parameters were verified. On one hand, the effect of growing ionic strength, in this case, growing NaCl concentration effect on the hydrolysis of ATP by NSP5 was analyzed. The hydrolysis of ATP was hardly affected by the presence of NaCl until very high concentrations of NaCl (0.4 M) (figure 8B). On the other hand, the effect of NaCl on the binding of ATP was determined. 8-Azido [$\alpha$-$^{32}$P]-ATP was used for this purpose. 8-Azido [$\alpha$-$^{32}$P]-ATP is an ATP analog, whose photocrosslinking to NSP5 was induced through UV light exposure. In this case, NSP5 was shown to be able to bind ATP also in high concentrations of NaCl and only in

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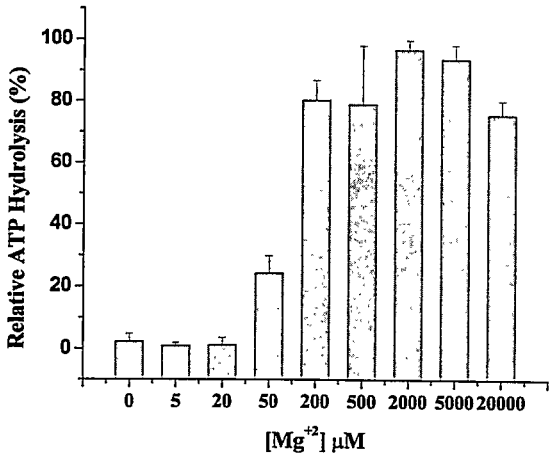


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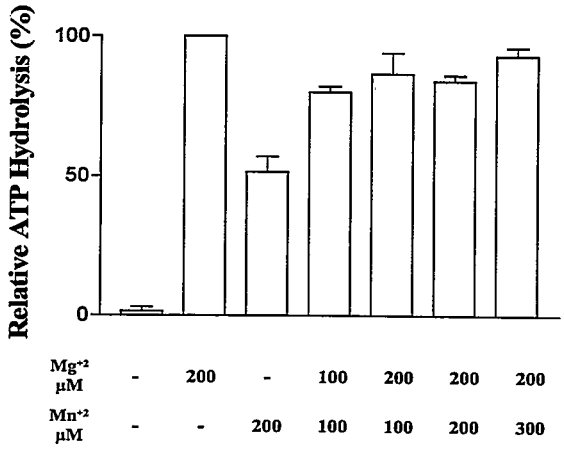
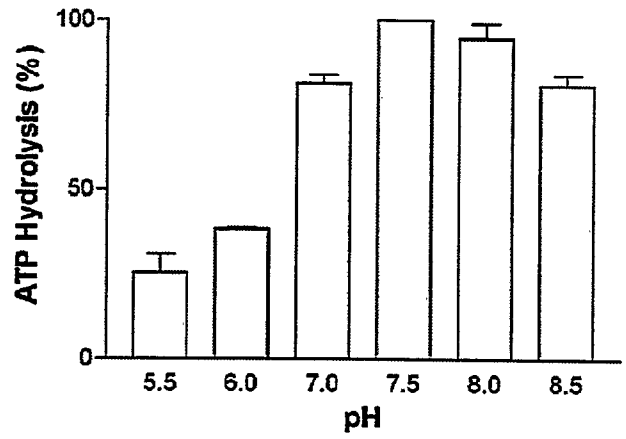



**Figure 7: ATPase activity characteristics – Divalent Cation.**

**Figure 7: ATPase activity characteristics – Divalent Cation.** (A) Reaction mixtures containing [$\alpha$-$^{32}$P]-ATP 0.833 μM, NSP5 4.67 μM, 50 mM Tris-HCl and NaCl 150 μM were incubated for 1 hour at 37°C with a final concentration of 200 μM divalent cation or as indicated. The highest hydrolysis was obtained when $Mg^{+2}$ was added to the reaction mixture. (B) Reaction mixtures were incubated with growing concentrations of $Mg^{+2}$. At $Mg^{+2}$ concentrations between 200 and 5000 μM the reaction was more efficient. (C) When $Mn^{+2}$ and $Mg^{+2}$ were combined at different final concentrations as indicated, no synergic effect of $Mn^{+2}$ was detected.

A

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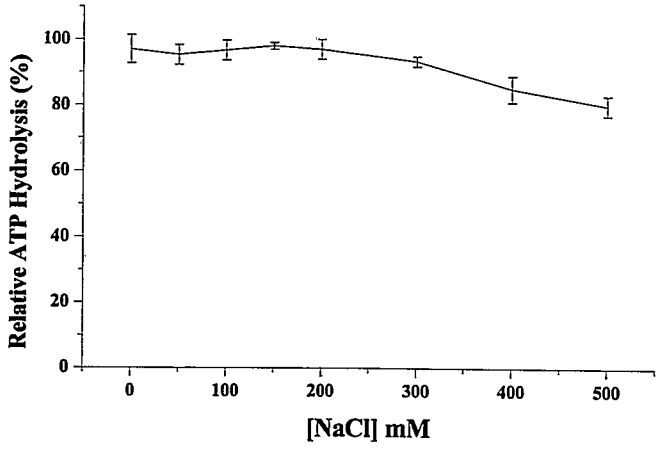


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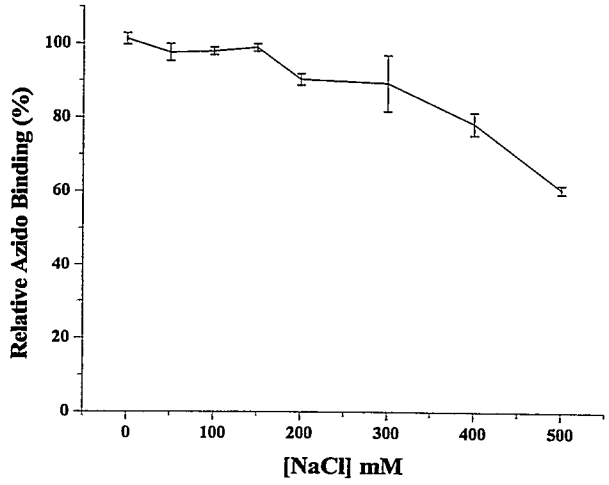



**Figure 8: ATPase activity characteristics – pH and Ionic Strength.**

**Figure 8: ATPase activity characteristics – pH and Ionic Strength.** (A) Reaction mixtures containing [α-$^{32}$P]-ATP 0.833 μM, 200 μM $Mg^{+2}$, NSP5 4.67 μM and NaCl 150 μM were incubated for 1 hour at 37°C in pH as indicated. The highest hydrolysis was obtained when the pH of the reaction mixtures was between 7 and 8. (B) Reaction mixtures containing [α-$^{32}$P]-ATP 0.833 μM, 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$ and NSP5 4.67 μM were incubated for 1 hour at 37°C in growing concentration of NaCl. An effect on ATP hydrolysis was detected in concentrations higher than 350 mM. (C) Reaction mixtures containing 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, NSP5 4.67 μM and 0.22 μM 8-Azido [α-$^{32}$P]-ATP were incubated for 1.5 minutes on ice under 254 nm UV light exposure in growing concentration of NaCl. A slight decrease in 8-Azido [α-$^{32}$P]-ATP binding was only detected at concentrations of NaCl higher than 300 mM.

very high concentrations of salt (higher than 0.3 M) ATP binding seemed affected (figure 8C).

### 5. Hydrolytic nucleotide preference

NSP2 was characterized as an NTPase that hydrolyze indistinguishably nucleotides without base preference. The discrimination of NSP5 for the hydrolysis of a particular nucleotide was evaluated. Competition experiments that allowed a comparison of the hydrolysis of different nucleotides by NSP5 were performed. When [$\alpha$-$^{32}$P] ATP was diluted with cold ATP, as expected, the levels of hydrolysis dropped promptly (figure 9). In contrast, when [$\alpha$-$^{32}$P] ATP was competed against with either GTP or UTP (Figure 9), the drop in hydrolysis was lower and almost undetectable until the ratio GTP or UTP: [$\alpha$-$^{32}$P] ATP was higher than 1000. Thus NSP5, differently than NSP2, has the ability to hydrolyze specifically ATP and does not hydrolyze to the same extent and in the same preference degree all nucleotides.

### 6. Characterization of kinetic parameters

Reaction mixtures containing the substrate [$\alpha$-$^{32}$P] ATP were incubated for the indicated time at the standard conditions (see materials and methods). Reactions were stopped adding EDTA, and immediate phenol-chloroform extraction was performed at the indicated times and spotted on a TLC plate.

Incubation of NSP5 with ATP resulted in a linear increase in the kinetics of product formation with a slight decrease in the rate after around 30 minutes (figure 10A).

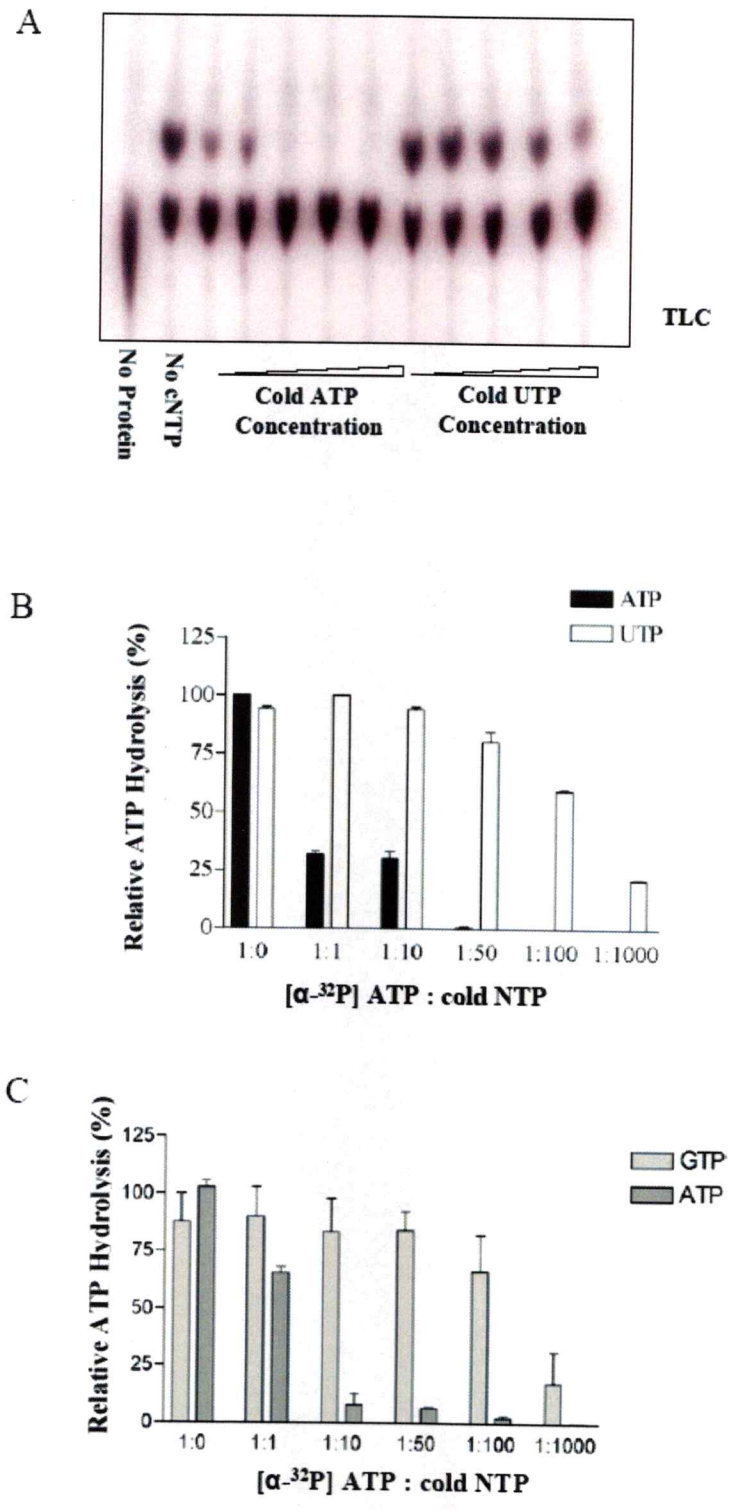
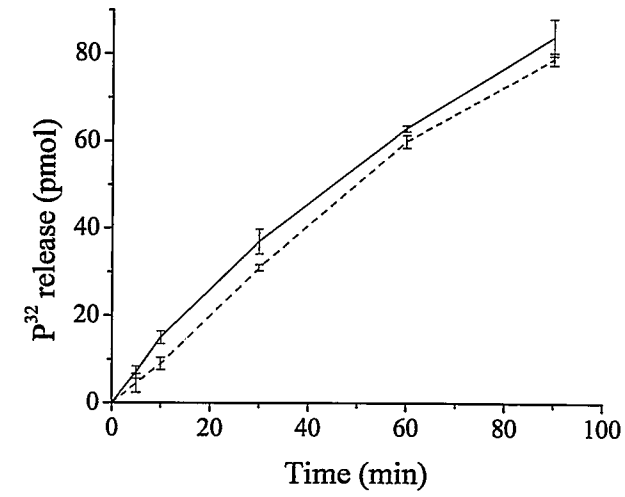



**Figure 9: Specificity of nucleoside triphosphate hydrolysis – Competition assay.**

**Figure 9: Specificity of nucleoside triphosphate hydrolysis – Competition assay.** Reaction mixtures containing [$\alpha$-$^{32}$P]-ATP 0.833 μM, 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, 150 mM NaCl and NSP5 4.67 μM and increasing concentrations of cold nucleotides as indicated were incubated 1 hour at 37°C. After incubation the reactions were analyzed through TLC and quantified using Phosphoimager. The competitions were performed with cold ATP and UTP ((A)-TLC and (B)-quantification) or cold ATP and GTP ((C)- quantification).



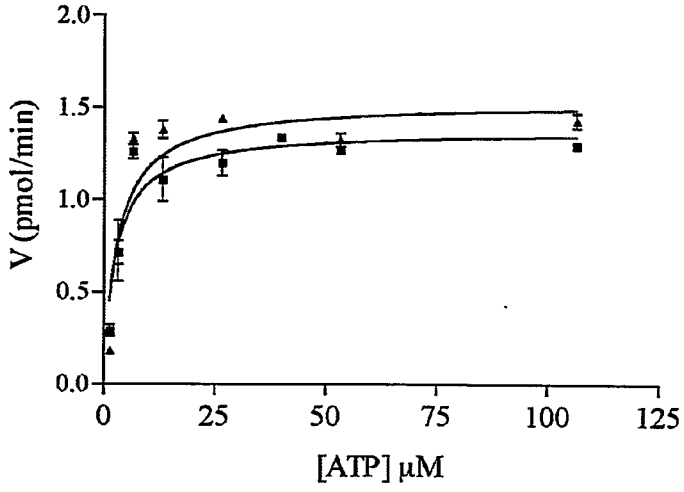




**Figure 10: Kinetics of ATP hydrolysis.** Reactions mixtures containing 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, 150 mM NaCl, 4.67 μM NSP5 and 6.67 μM [γ-$^{32}$P] ATP were incubated at 37°C for the indicated periods of time. ATP hydrolysis was monitored by analyzing the reaction mixture through TLC and quantifying with the Phosphoimager (A). Reaction mixtures containing 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, 150 mM NaCl, 4.67 μM NSP5 and increasing concentrations of [γ-$^{32}$P] ATP were incubated for 30 minutes. The hydrolysis was monitored through TLC and quantified with a Phosphoimager. The rate of $^{32}$P release (pmol/min) was plotted as a function of [γ-$^{32}$P] ATP concentration (B). Each curve represents a different protein preparation.

Addition of increasing amounts of ATP resulted in a concentration dependent increase in the hydrolysis between 0 and 25 μM ATP. The rate of ATP hydrolysis was measured in ATP concentrations ranging between 0 and 120 μM allowing the determination of Vmax of hydrolysis as 21 fmol product/min and Km as 2.9 μM (figure 10B; table 2). These constant values are similar to those described for the NTPase activity of NSP2 *wt* determined previously (Vasquez del Carpio *et al.*, 2004).

### 7. Effect of the presence of NSP2 on the hydrolysis of ATP by NSP5

In mammalian reoviruses, the core protein μ2 has an NTPase activity which is stimulated by its interaction with λ3, the RNA dependent RNA polymerase of reoviruses (Kim *et al.*, 2004). As reported previously, NSP2 and NSP5 are the viral proteins required for the formation of viroplasms and NSP2 has been shown to have a stimulating effect on the phosphorylation of NSP5, both *in vivo* and *in vitro* (Afrikanova *et al.*, 1998; Carpio *et al.*, 2004; Vasquez del Carpio *et al.*, 2004; Vende *et al.*, 2002). To explore the effect of NSP2 on the ATPase activity of NSP5, the hydrolysis of ATP was measured for each of the two enzymes or in combination of both (figure 11). The hydrolysis when both proteins were present in the reaction mixture was similar to the addition of the hydrolysis obtained for each protein alone suggesting that the total hydrolysis corresponds to the catalytic activity of each protein in the reaction mixture (figure 11).

To further determine whether the presence of NSP2 had any stimulatory effect on NSP5 hydrolysis of ATP, a mutant of NSP2 with a substitution of the Histidine 225 for an Alanine was added to the a reaction mixture. This mutant was previously

**Table 2: Comparison between the kinetics parameters of ATP hydrolysis of NSP2, NSP5 and DnaK.**

| Protein | Vmax | Km |
|---|---|---|
| **NSP5 (Rotavirus)** | 21 (fmol/min/pmol enzyme) | 2.9 μM |
| **NSP2 (Rotavirus) (Vasquez del Carpio *et al.*, 2004)** | 18 (fmol/min/pmol enzyme) | 0.65 μM |
| **DnaK (Barthel *et al.*, 2001)** | 0.16 (pmol/min/pmol enzyme) | 27.5 nM |

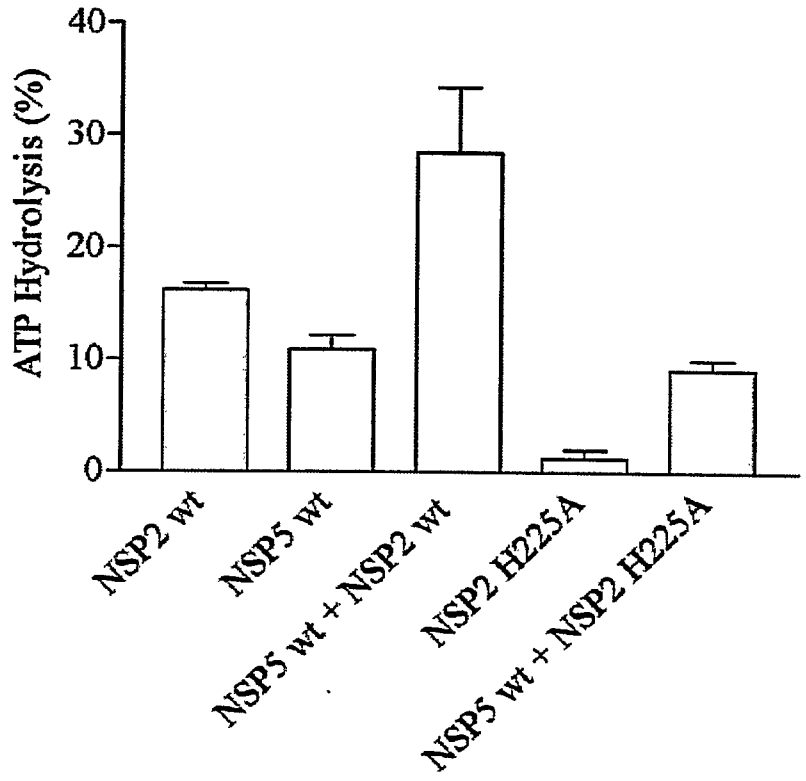



**Figure 11: ATPase activity of NSP5 in the presence of wild type NSP2 and H225A mutant NSP2.** The purified *wt* NSP2 or H225A NSP2 were added to reaction mixtures containing 0.833 μM [α-$^{32}$P]-ATP, 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, 150 mM NaCl with or without NSP5 and incubated for one hour at 37°C. [γ-$^{32}$P] ATP hydrolysis was determine by TLC and quantified with Phosphoimager. Final concentrations of *wt* NSP2 or H225A NSP2 were 1.87 μM and of *wt* NSP5 were 2.33 μM.

characterized in the laboratory as highly affected in its NTPase activity (Vasquez del Carpio *et al.*, 2004). The total hydrolysis of the reaction mixture containing *wt* NSP5 and H225A NSP2 was similar to the hydrolysis of NSP5 alone (figure 11). Thus, neither the NTPase activity of NSP2, nor its presence had an effect on the ATPase activity of NSP5.

### 8. Effect of RNA on the ATPase activity of NSP5

NSP5 has been reported to bind both, ssRNA and dsRNA, with similar affinity in non specific manner (Vende *et al.*, 2002).

- Effect of ssRNA

In some ATPases, UTPases and NTPases, the rate of hydrolysis is affected by the presence of ssRNA (Colletti *et al.*, 2004; Warrener *et al.*, 1993). To determine whether NSP5 ATP hydrolysis is affected by the presence of ssRNA, either a sequence specific ssRNA from gene 8 of rotaviruses or a random sequence of ssRNA-poly U, ssRNAs were added to the reaction mixtures and the hydrolysis was assessed by TLC (figure 12A). Neither a sequence specific ssRNA nor a random sequence ssRNA in different concentrations seemed to have an effect of the hydrolysis of ATP (figure 12B).

- Effect of dsRNA

A sequence specific dsRNA corresponding to gene 2 was added to reaction mixtures in growing concentrations and the hydrolysis was compared to that in the absence of RNA (Figure 12C). A slight increase in the hydrolysis was observed when dsRNA was added and as its concentration increased.

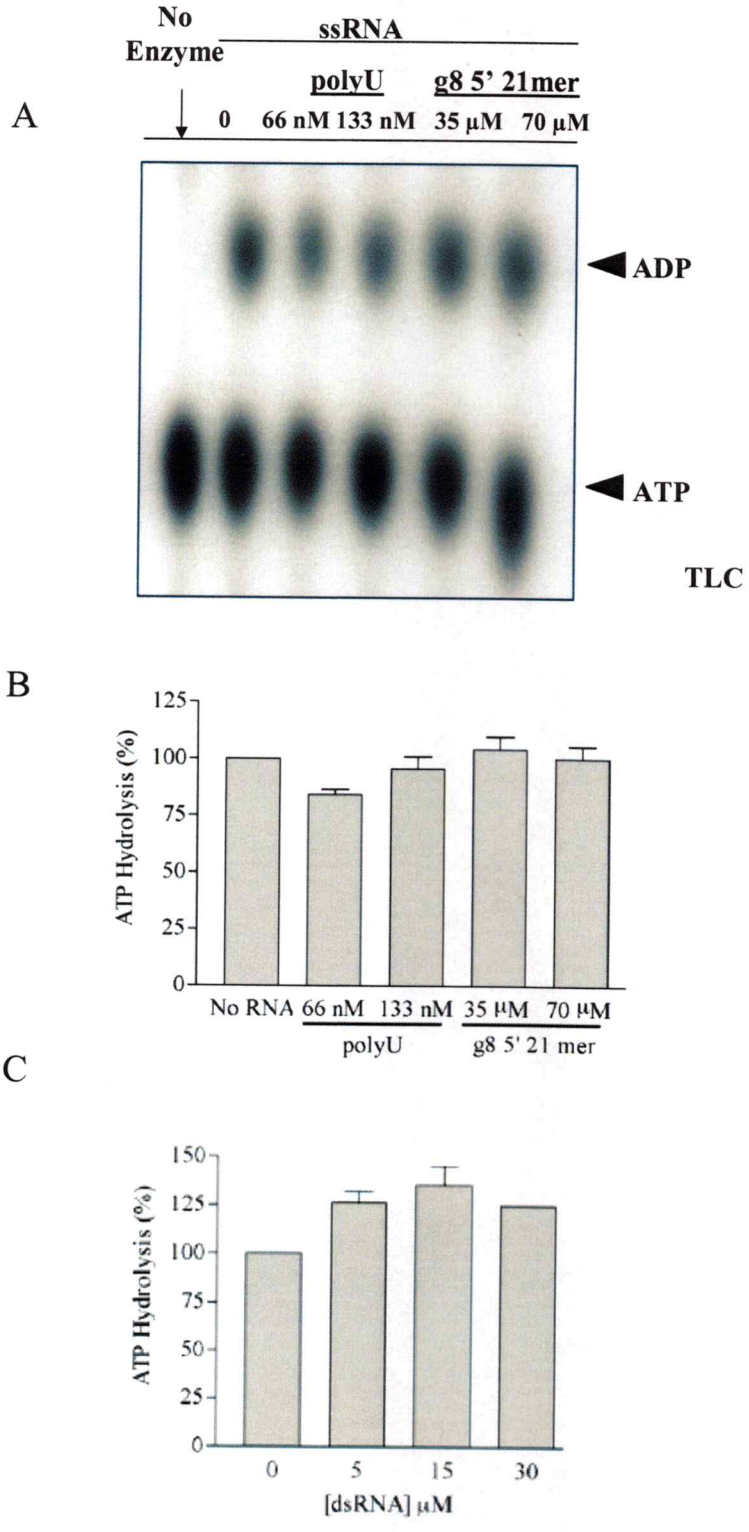



**Figure 12: Effect of ssRNA and dsRNA on ATP hydrolysis.**

**Figure 12: Effect of ssRNA and dsRNA on ATP hydrolysis.** Sequence specific ssRNA or PolyU in final concentrations as indicated were added to reaction mixtures containing [$\alpha$-$^{32}$P]-ATP 0.833 $\mu$M, 50 mM Tris-HCl pH 7.5, 200 $\mu$M $Mg^{+2}$, 150 mM NaCl and 4.67 $\mu$M NSP5 ((A)-TLC and (B)-Quantification). Sequence specific dsRNA was added to reaction mixtures in growing concentrations as indicated (C). The ATP hydrolysis was analyzed through TLC and quantified with a Phosphoimager.

### 9. Model of NSP5- Putative site of ATP binding

As mentioned before, the structure of NSP5 has not been solved. Its sequence is extremely conserved among strains of group A rotaviruses and is also highly conserved when compared with group B and group C of rotaviruses as well as avian sequences.

In order to identify and to mutate the ATP binding site of NSP5 we modeled the protein a molecular dynamic methodology. The first step was to model the structure of NSP5. Part of NSP5 sequence showed some identity with a protein with solved and published structure, a ribonuclease, hybrid between ribonuclease A and a bovine ribonuclease. This structure had more than 30% identity with part of the sequence of NSP5 and served to model, based on homology, the last 2/3 of NSP5 (from aminoacid 65 to the C-termini). Next, docking technique was used in order to find the aminoacids that would be critical for the binding of ATP to NSP5.

Three amino acids were proposed as critical in the process of hydrolysis of ATP (figure 13), Arg 144, Lys 145 and Arg 186. This amino acids were mutated through site directed mutagenesis and compared to *wt* NSP5 in their abilities to bind ATP (through azido binding assays), to hydrolyze ATP (ATPase assays analyzed through TLC) and to undergo autophosphorylation and hyperphosphorylation mediated through NSP2 *in vitro*.

- Phosphorylation of *wt* and mutant NSP5

As mentioned previously, *in vitro*, NSP5 undergoes autophosphorylation and is hyperphosphorylated in the presence of NSP2 (Afrikanova *et al.*, 1998; Vasquez del Carpio *et al.*, 2004; Vende *et al.*, 2002). In order to analyze the effect of the mutations performed on NSP5 according to the bioinformatic model phosphorylation assays of

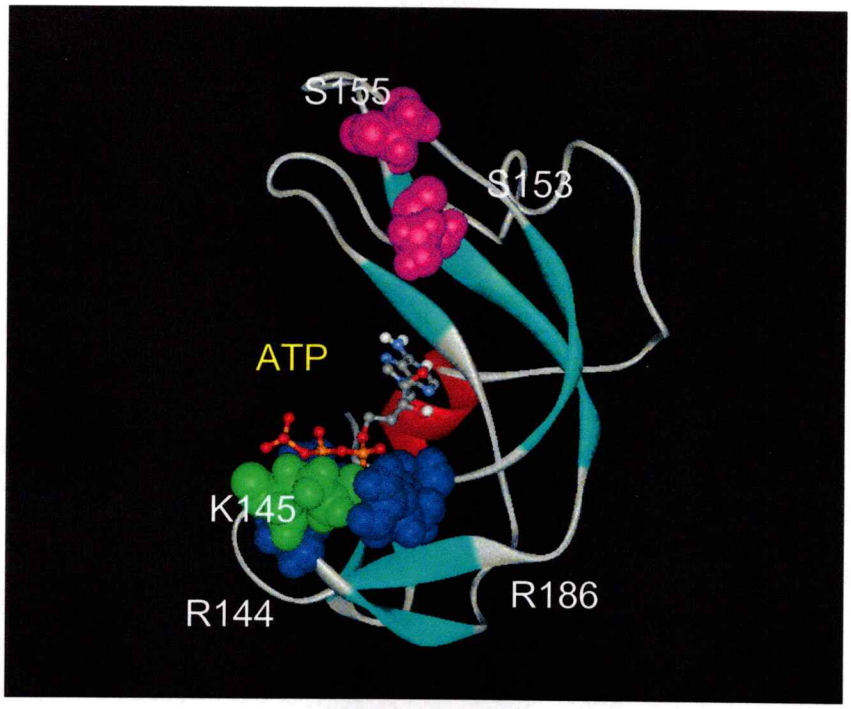



**Figure 13: Model of the structure of NSP5.** The sequence of NSP5 from SA11 strain was analyzed in the PDB (GCG) database and a protein with a similarity higher than 30% with NSP5 sequence was found. This similarity allowed the construction of a model of NSP5 through homology. Following the construction of the model with the program *MODELLER*, a relaxation using the program *CHARMm28* was performed. The program *AUTODOCK 3.02* was used to localize the putative ATP binding site and three amino acids were identified as possible target that could affect the ATP binding, Arg 144, Lys 145 and Arg 186.

NSP5 were performed *in vitro* with NSP5 alone or in the presence of NSP2. The autophosphorylation of NSP5 *wt* was similar to that of mutants of NSP5 (figure 14A and 14B). The mutations also did not seem to have a significant effect on the higher extend of phosphorylation of NSP5 in the presence of NSP2 (figure 14C).

- Azido ATP binding of *wt* NSP5 and mutant NSP5

In order to analyze a possible effect of the mutations on the ability of NSP5 to bind ATP an analog of ATP, 8-Azido-[$\alpha$-$^{32}$P] ATP, that undergoes photocrosslinking induced by UV, was incubated with *wt* and mutant NSP5. There was no major difference between the binding of the mutant NSP5 in comparison to *wt* NSP5 (figure 14D).

- ATP hydrolysis of *wt* and mutant NSP5

ATPase activity of *wt* and mutant NSP5 was compared through hydrolysis assays and TLC analysis. The ATP hydrolysis of the mutants R144A and K145A was similar to that of *wt* NSP5. The mutant R186A was slightly affected in the ATP hydrolysis (figure 15).

### 10. Model of NSP5- Putative site of $Mg^{+2}$ binding

The ATPase activity of NSP5 was shown to be dependent on the presence of $Mg^{+2}$. Thus, through molecular dynamic simulation the binding site of $Mg^{+2}$ was identified based on the model of the structure of NSP5 previously mentioned and taking into consideration that only a mutation of the aminoacid R186 had an effect on the ATPase activity of NSP5. Two aminoacids, E151 and D153, were proposed as the putative sites of $Mg^{+2}$ binding (figure 16). These amino acids were mutated

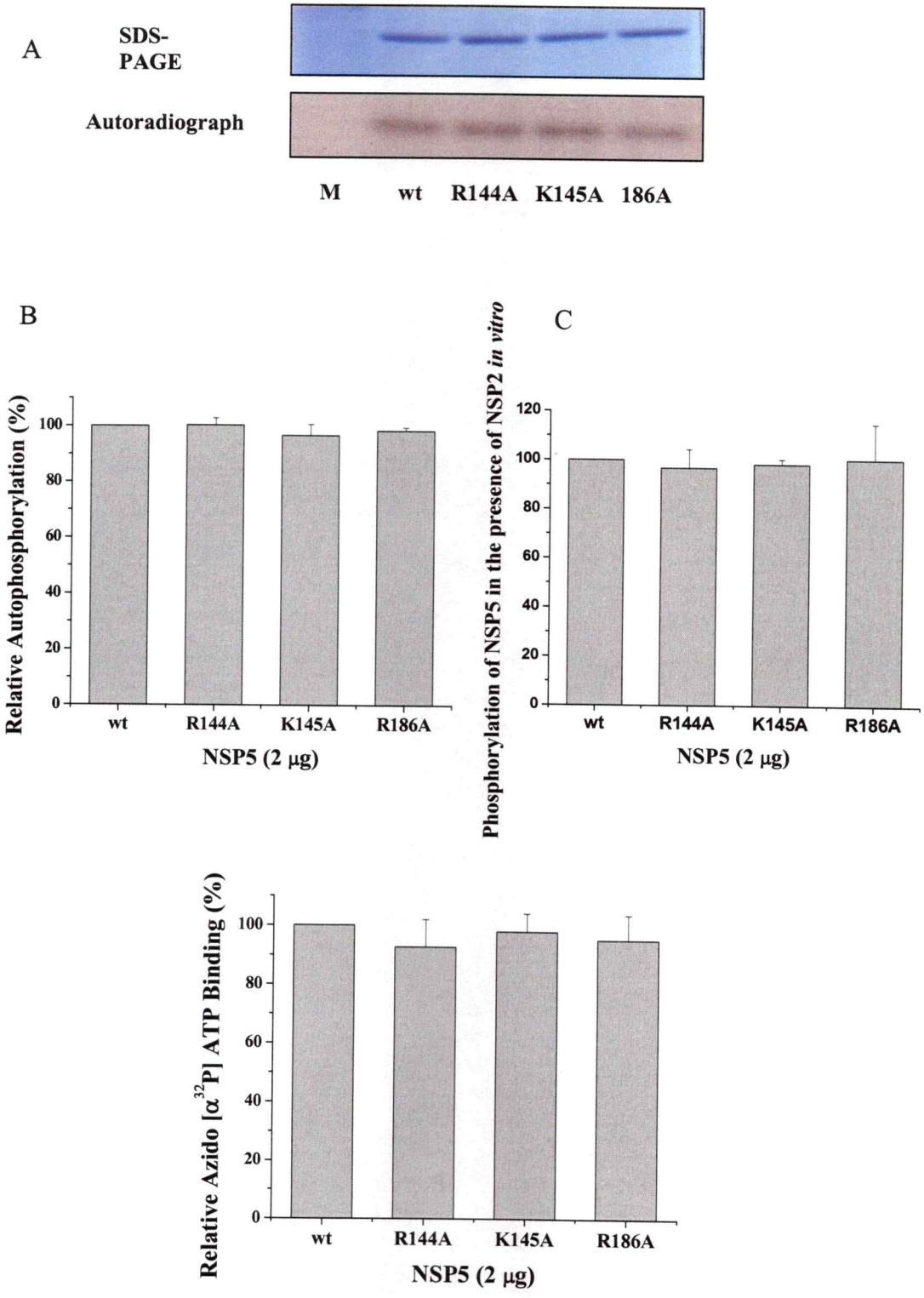



**Figure 14: Phosphorylation of *wt* and mutant NSP5 *in vitro*.**

**Figure 14: Phosphorylation of *wt* and mutant NSP5 *in vitro*.** Reaction mixtures containing 4.67 μM NSP5, 150 mM NaCl, 120 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$ and 3.33 pmol [γ-$^{32}$P]-ATP were incubated for 2 hours at 37°C. (A) NSP5 *wt* and mutants were solved through an SDS-PAGE, Coomassie stained (*up*) or analyzed through autoradiography (*down*).(B) NSP5 *wt* and mutant were incubated for 2 hours, solved through SDS-PAGE, quantified with a Phosphoimager (C) NSP5 *wt* and mutant were incubated with 1.87 μM *wt* NSP2 for 2 hours, solved through SDS-PAGE, quantified with a Phosphoimager. (D) Reaction mixtures containing 4.67 μM *wt* or mutant NSP5, 150 mM NaCl, 120 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$ and 0.22 μM 8-Azido-[α-$^{32}$P]-ATP were incubated for 1.5 min on ice and exposed to 254 nm UV light . Proteins were solved through SDS-PAGE, quantified with a Phosphoimager.

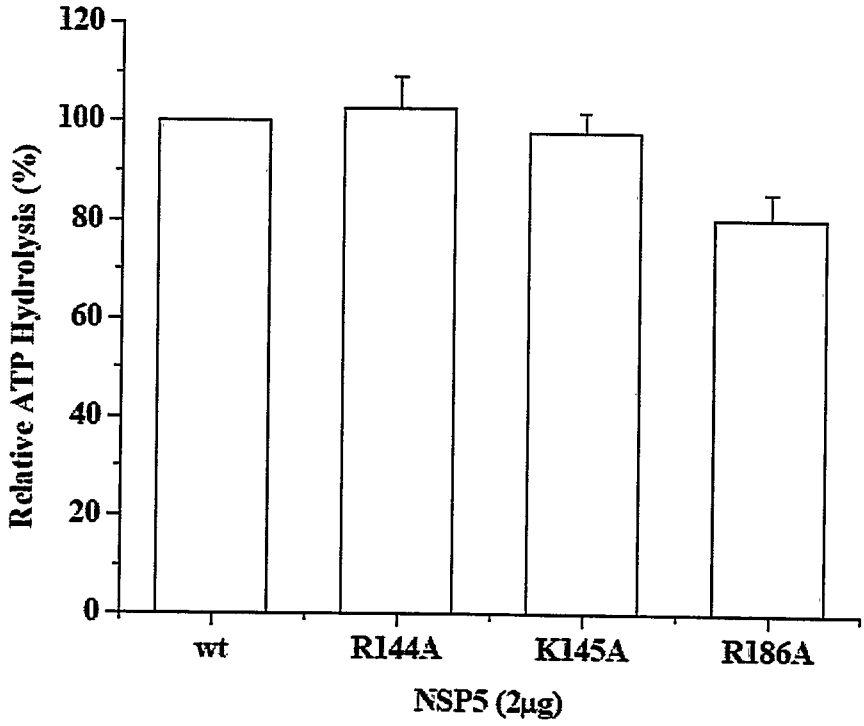



**Figure 15: Comparison of the ATPase activity of NSP5 *wt* and mutants.** [α-$^{32}$P] ATP hydrolysis in reaction mixtures containing [α-$^{32}$P]-ATP 0.833 μM, 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$, 150 mM NaCl and 4.67 μM NSP5, incubated for one hour at 37°C was detected by TLC and autoradiography. A slight decrease in ATP hydrolysis of R186A NSP5 is detected.

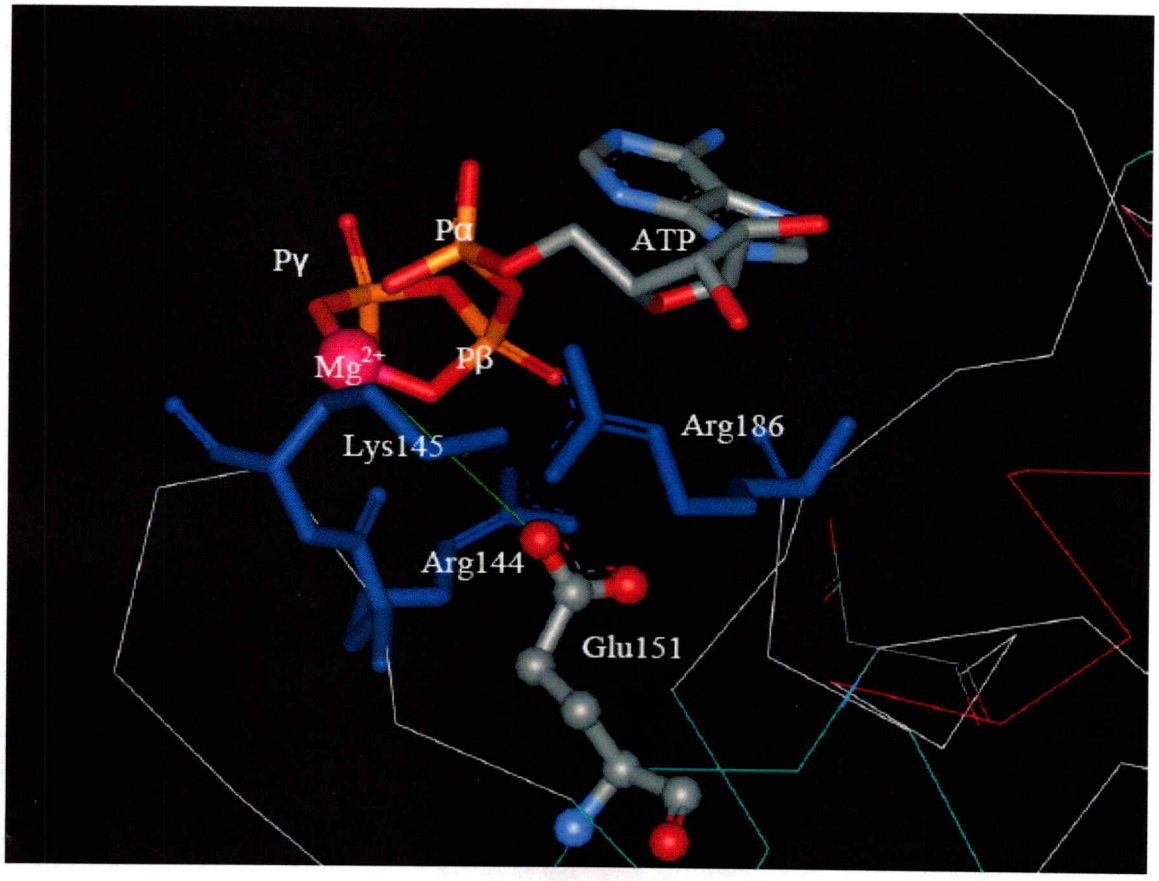



**Figure 16: Model-Putative site of $Mg^{+2}$ binding.** The determination of putative amino acids crucial for $Mg^{+2}$ binding was identified following results indicating this cation was essential for the ATPase activity. Based on this model the putative amino acids critical for the cation binding were Glutamic Acid 151 and Aspartic Acid 153.

simultaneously using site directed mutagenesis and the ATPase activity of the double mutant NSP5 was analyzed. When compared to *wt* NSP5, no major effect on the ATPase activity of the double mutation was detected (figure 17).

### 11. Stability of the *in vitro* phosphorylated isoform of NSP5

When incubated with [$\gamma$-$^{32}$P] ATP, NSP2 was shown to be phosphorylated *in vitro*, although no phosphorylated NSP2 was detected *in vivo*. Further studies determined NSP2 phosphorylation was the consequence of the formation of a stable intermediate of a nucleotide hydrolysis reaction, a phosphorylated histidine through a phosphoramidate bond (Vasquez del Carpio *et al.*, 2004). NSP5 is considered a phosphoprotein which has an autokinase activity enhanced in the presence of NSP2 *in vitro* and undergoes further degrees of phosphorylation *in vivo* where the presence of NSP2 has been shown to be essential (Afrikanova *et al.*, 1998). The hyperphosphorylation of NSP5 *in vivo* was shown previously to be on serine residues (Afrikanova *et al.*, 1996; Blackhall *et al.*, 1998; Eichwald *et al.*, 2002; Poncet *et al.*, 1997). In order to determine whether the autophosphorylation of NSP5 detected *in vitro* was a result of a phosphoramidate (P-N) bonded phosphate or a phosphoester bonded phosphate (P-O) the stability of the phosphoamino acid under acid or alkaline conditions was analyzed. Autophosphorylation assays where performed. Briefly, NSP5, and NSP2 as a control, were incubated in parallel for 2 hours at 37°C with [$\gamma$-$^{32}$P] ATP; the proteins were solved by SDS-PAGE, transferred to a PVDF membrane and subsequently treated for 2 hours at 65°C with either 1 M KOH or 6 M HCl or air dried without treatment. After the acid/alkaline treatment or no treatment, the proteins were identified by

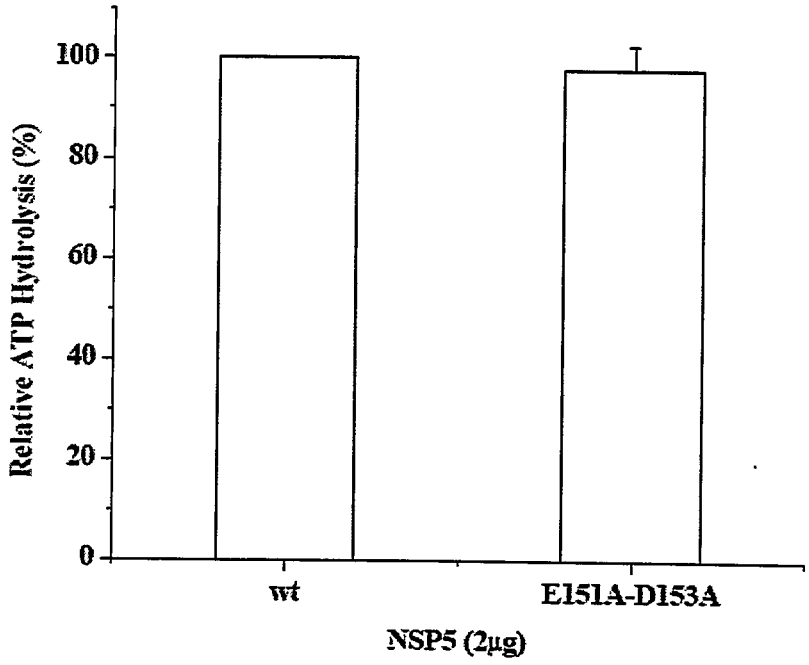



**Figure 17: Comparison of the ATPase activity of *wt* and E151A-D153A mutant NSP5.** [$\alpha$-$^{32}$P] ATP hydrolysis in reaction mixtures containing [$\alpha$-$^{32}$P]-ATP 0.833 µM, 50 mM Tris-HCl pH 7.5, 200 µM $Mg^{+2}$, 150 mM NaCl and 4.67 µM NSP5 *wt* or mutant, incubated for one hour at 37°C, was detected by TLC and autoradiography.

autoradiography. Phosphoester bonds (P-O) are susceptible to alkaline conditions and stable under acid conditions while phosphoramidate bonds (P-N) are susceptible to acid conditions and stable under alkali conditions (Figure 18). Our results suggests NSP5 is phosphorylated through a phosphoramidate linkage similarly to the case of NSP2 previously reported (Vasquez del Carpio *et al.*, 2004). Thus, *in vitro* the phosphorylation is not on a serine residue (a phosphoester bond), in contrast to what was previously reported *in vivo*.

**12. Effect of amino acid substitution of serine 67 on the ATPase activity of NSP5**

NSP5 serine 67 has been proposed to be essential for the phosphorylation *in vivo*, suggested to be the first amino acid phosphorylated in a cascade of events rendering the protein hyperphosphorylated (Eichwald *et al.*, 2004). A model have been proposed, based on oligopeptide assays, suggesting serine 67 is phosphorylated by a cellular kinase, Casein Kinase I (CKI), after a structural modification, through the interaction with NSP2, which allows this amino acid to be exposed and thus, phosphorylated (Eichwald *et al.*, 2004). In order to determine whether there is any involvement of S67 in the ATPase activity observed for NSP5 a site directed mutagenesis of the enzyme was performed mutating S67 to an alanine or to an aspartic acid that mimics the negative charge of a phosphorylated amino acid. The autophosphorylation of *wt* NSP5 in comparison to the S67 mutants was analyzed and no significant difference was observed (Figure 19). Both, *wt* NSP5 and the mutants in S67 showed no significant difference in the hydrolysis of ATP (Figure 20) suggesting S67 is not essential in the autophosphorylation *in vitro* as well as in the ATPase activity of NSP5.

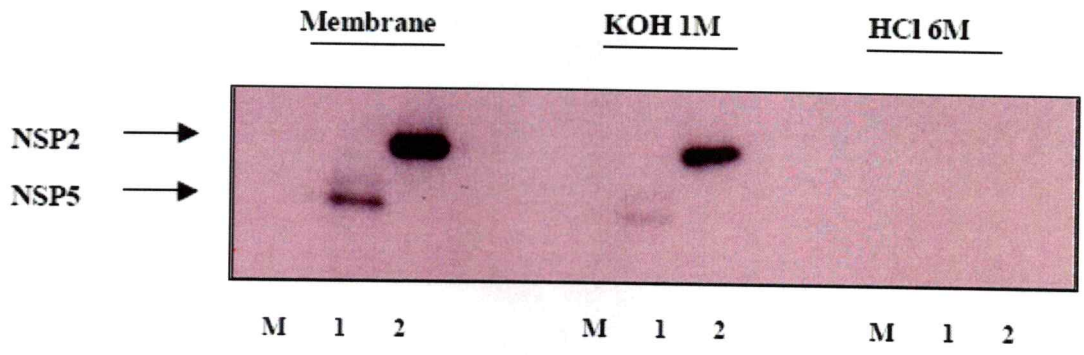



**Figure 18: Hydrolytic Stability of the phosphoamino acid in NSP5 under Acid or Alkaline Conditions.** Reaction mixtures for NSP5 and NSP2 autophosphorylation contained 2 µg of NSP5 or 1 µg of NSP2 and 10µCi of [$\gamma$-$^{32}$P] ATP and were incubated for 2 h at 37 °C. Phosphorylated proteins were solved through SDS-PAGE and transferred to a PVDF membrane. Membranes were autoradiographed directly or after an individual incubation at 65 °C for 2 h in alkaline (1 M KOH) or acid (6 M HCl) conditions. M- Molecular marker, 1- NSP5 and 2- NSP2.

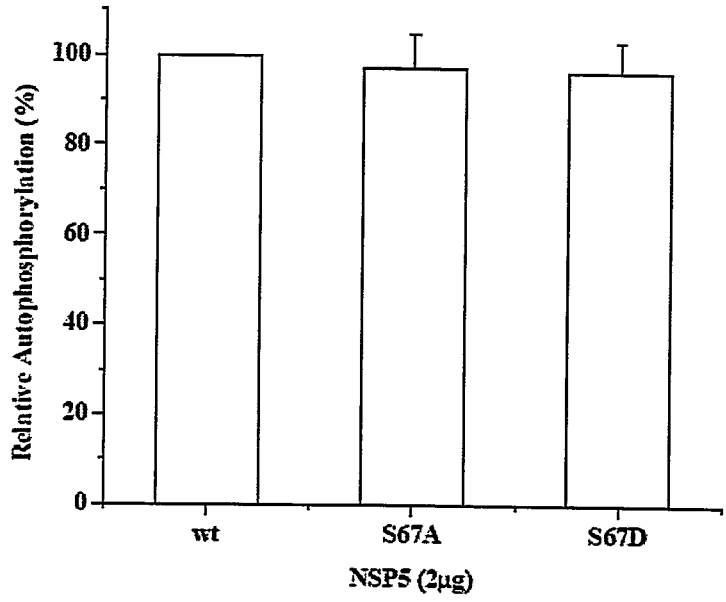



**Figure 19: Autophosphorylation of *wt* and S67A, S67D mutants.** Reaction mixtures containing [$\alpha$-$^{32}$P]-ATP 0.833 µM, 200 µM $Mg^{+2}$, 150 mM NaCl, 4.67 µM NSP5 and 120 mM Tris-HCl pH 7.5 were incubated for 2 hours at 37°C.

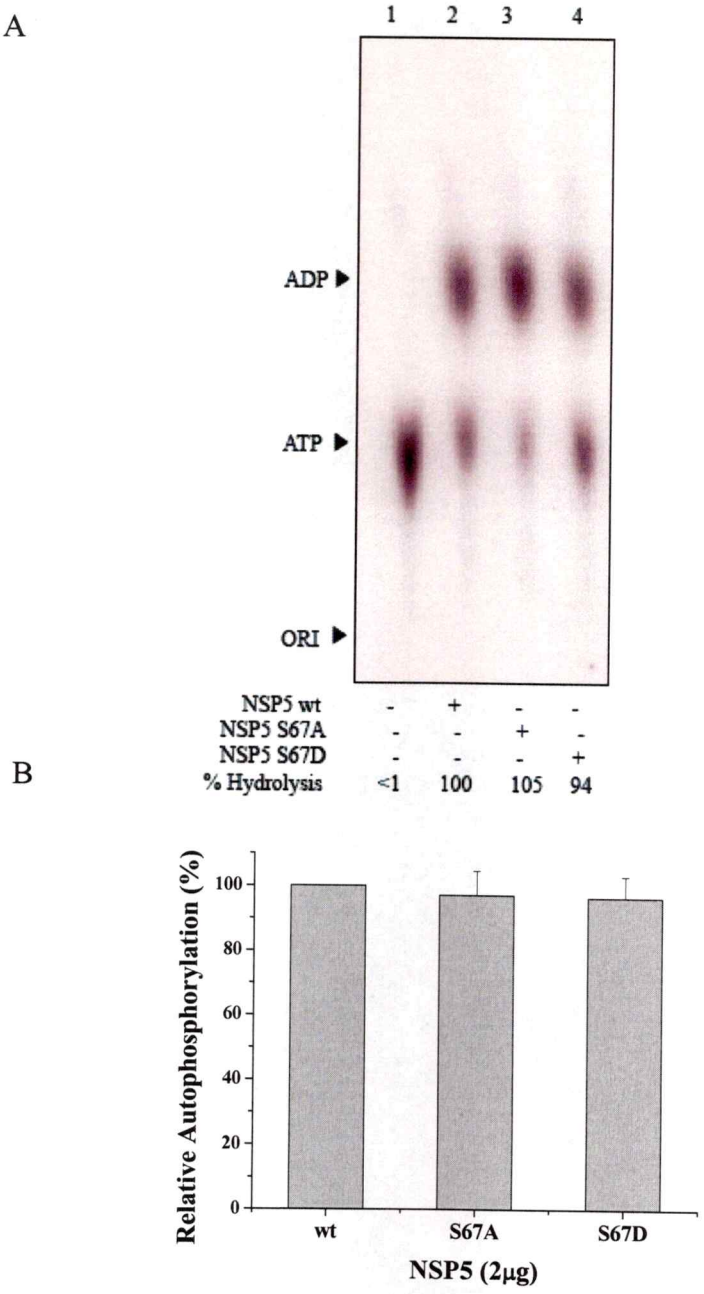



**Figure 20: ATP hydrolysis activity of NSP5 mutants.** Reaction mixtures containing 4.67 μM *wt* NSP5, S67A NSP5 or S67D NSP5, [α-$^{32}$P]-ATP 0.833 μM, 50 mM Tris-HCl pH 7.5, 200 μM $Mg^{+2}$ and 150 mM NaCl were incubated one hour at 37°C (A). The hydrolysis was analyzed through TLC and quantified with a Phosphoimager (B).

**13. Analysis of sequences of gene 11 obtained from field samples taken from infected patients and laboratory strains.**

In collaboration with other labs, samples of rotavirus from infected patients were obtained. The sequences of NSP5 and NSP2 from different genotypes of laboratory strains of rotavirus as well as from samples from patients were analyzed and compared. The viral aminoacid sequences were highly conserved, in particular the sequence of NSP5 (figure 21).

Since NSP2 and NSP5 have been proposed to interact in order to form the viroplasms, a search for changes in NSP2 sequences which had an effect on their NSP5 counterparts in the same strains in comparison to other strains was performed. When phylogenetically analyzed and the distances between themselves compared, the sequences of NSP2 and NSP5 showed a similar display of distances among strains (figure 22). Since the structure of NSP5 is unknown, a localization of the mutations on the structure of NSP2 was performed. In the strains in which NSP2 sequence changed on the surface of the structure, the sequence of NSP5 were analyzed and compared. No clear correlation between the changes in NSP5 and those on the sequence of NSP2 in the different strains analyzed was detected.

**14. Characterization of the second ORF of gene 11**

Gen 11 of rotaviruses is the only one among the 11 segments of rotaviruses that possesses a second ORF that codes for the smallest protein of rotavirus, NSP6, with unknown function. Interestingly, when the sequences of gene 11 were analyzed and

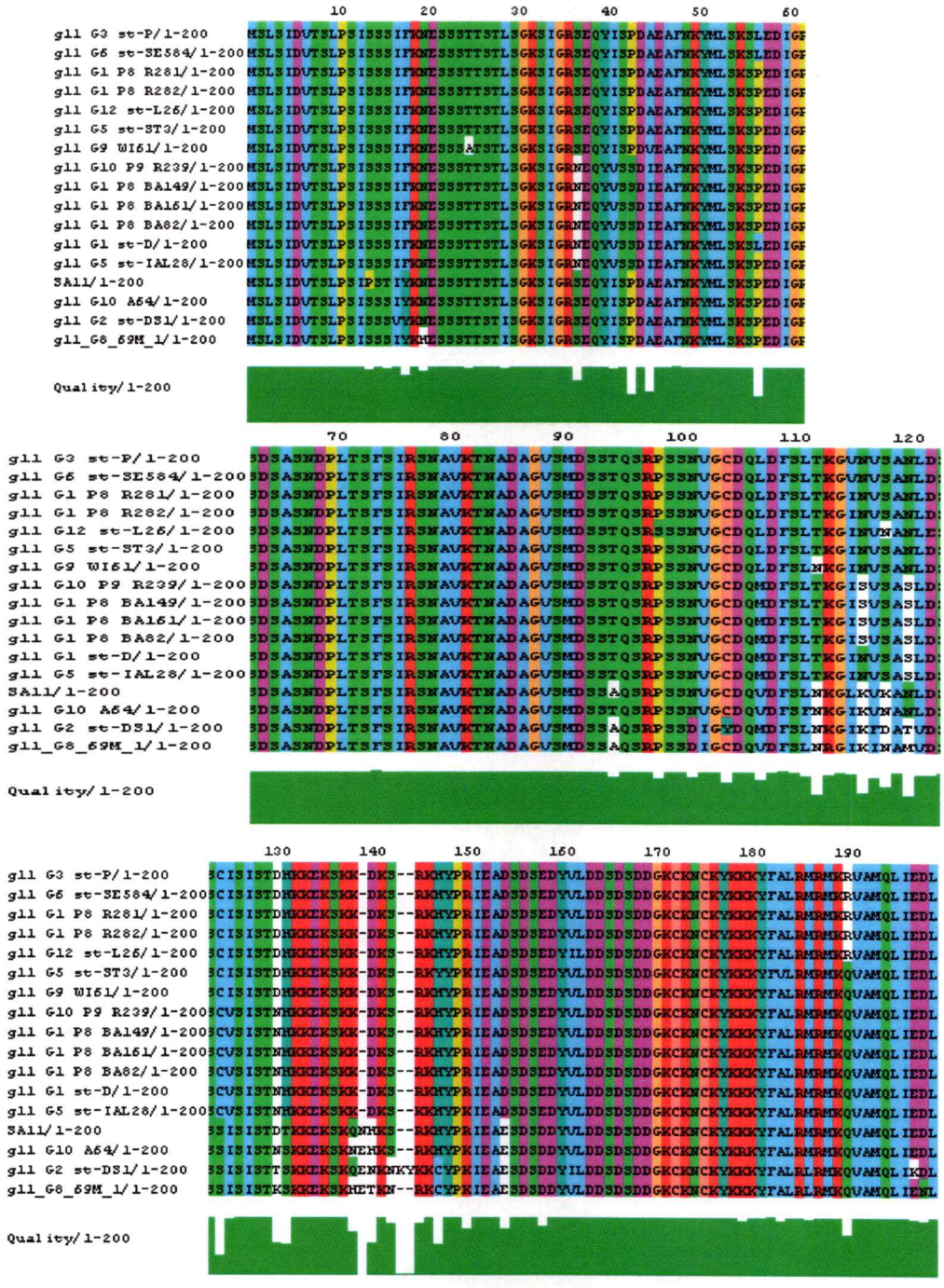


**Figure 21: High sequence conservation of NSP5 sequences in field samples of infected patient strains and from laboratory strains.** The sequences from laboratory strains and patients recovered samples were aligned using the program *ClustalW*.

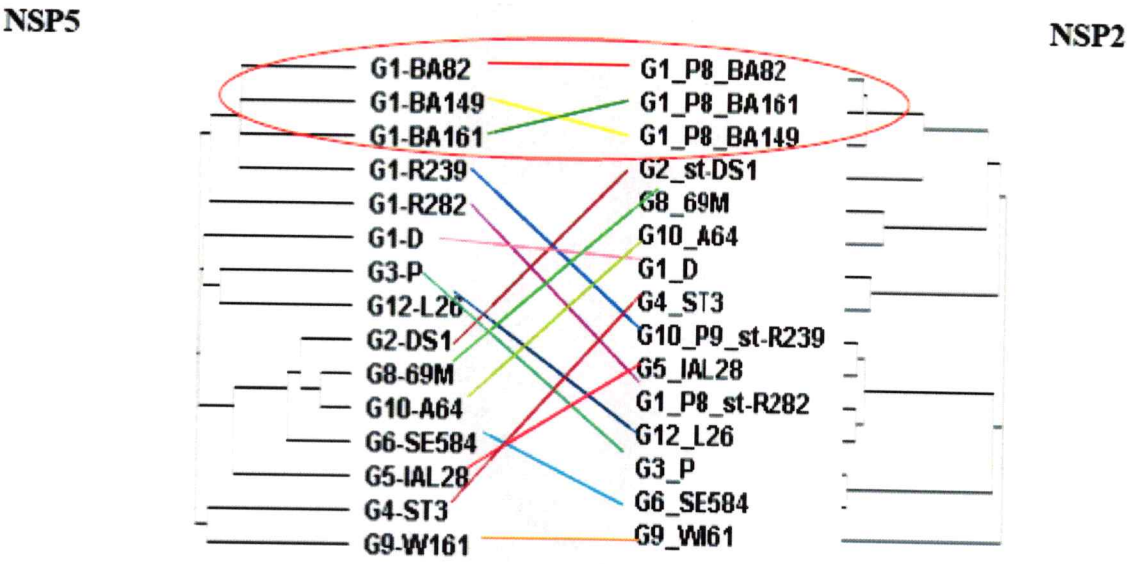



**Figure 22: Comparison between phylogenetic trees of NSP5 and NSP2 laboratory strains and field samples of patient strains sequences.** Phylogenetic trees of the amino acids sequences of both proteins, NSP2 and NSP5 were prepared using the program *ClustalW*. The sequences of both proteins from the same strain are connected with color lines. As highlighted, the sequences of both proteins were clustered similarly in both trees. When further analyzed, no clear pattern of changes has been found, thus not allowing a clear correlation between changes in both proteins in different strains.

compared among themselves and with other sequences in the database, we were able to detect a conservation of NSP6 ORF in strains collected from patients. In contrast, in some of the lab strains NSP6 ORF was lost (figure 23).

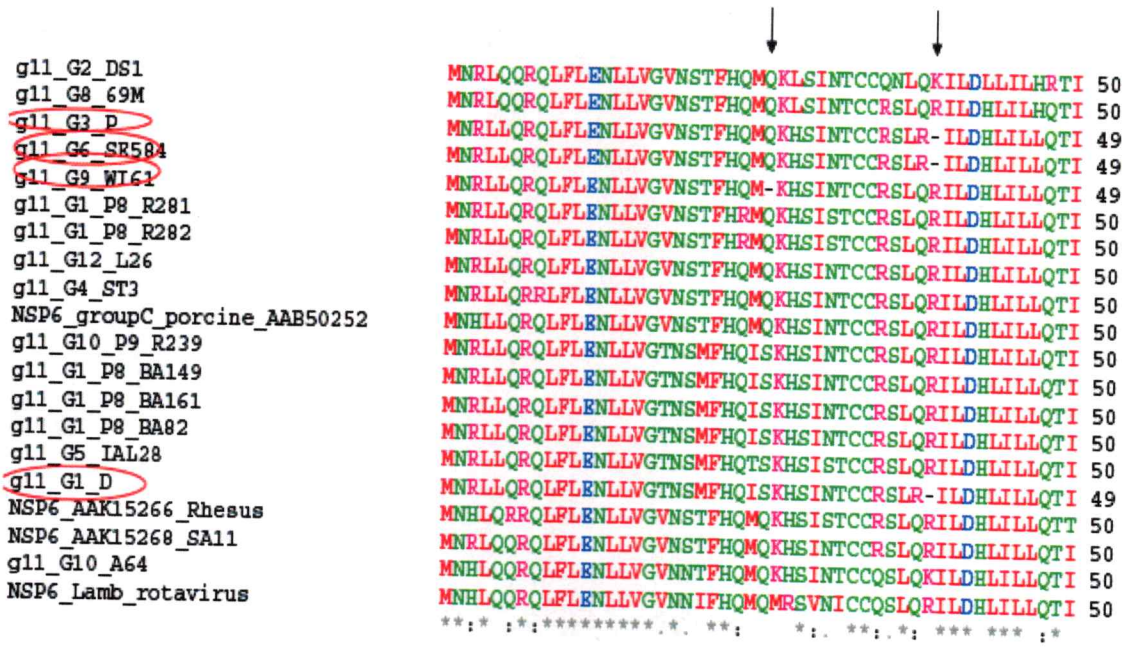



**Figure 23: Sequences from laboratory strains and field samples from patients with truncated sequences of NSP6.** Analysis using gene 11 sequences from either laboratory strains of rotavirus or samples collected from patients suffering from rotavirus infection. In arrows, stop codons in the NSP6 ORF. Marked with red circles, strains with truncated NSP6 ORFs, all of them, laboratory strains. Alignment performed with the program *ClustalW*.

## DISCUSSION

NTPases have been identified and characterized in several viruses of the *Reoviridae*. In the case of BTV, NS2 has been shown to have a nucleotidyl phosphatase activity able to hydrolyze γ, β and α phosphates from any NTP (Taraporewala *et al.*, 2001). Similarly, in avian reovirus, σA has also been shown to have a strong phosphatase activity cleaving all three phosphodiester bonds from any NTP (Yin *et al.*, 2002).

In the cores of mammalian reoviruses two proteins with NTPase and RTPase activities have been identified and characterized (Kim *et al.*, 2004; Noble and Nibert, 1997a, b). Although the role of both proteins, λ1 and μ2, has not been determined, it is believed they have different roles in the viral cycle since their NTPase activities have different biochemical characteristics.

NSP2 of rotaviruses has been identified as an NTPase that only hydrolyzes the γ phosphodiester bond of an NTP without any preference for the base of the NTP (Taraporewala *et al.*, 1999). When tested for NTPase activity, NSP5 showed the ability to hydrolyze the γ phosphate of ATP thus indicating a previously unknown enzymatic activity of NSP5.

Differently than other NTPases mentioned above, NSP5 has a preference for hydrolysis of ATP in comparison to other NTPs as tested in competition assays. The conditions for further analyzes were determined, such as pH, ionic strength and cation preference. The reaction was completely dependent upon the presence of a cation, in particular $Mg^{+2}$.

Several NTPases have been shown to be positively affected by the presence of nucleic acids (Colletti *et al.*, 2004; Warrener *et al.*, 1993). NSP5 has an unspecific sequence affinity for ssRNA and dsRNA (Vende *et al.*, 2002), although only the latter showed a slight positive effect on the ATPase activity of NSP5.

NSP2 has a positive effect on NSP5 phosphorylation both, *in vivo* and also *in vitro* in the absence of RNA. However, there was no effect, neither of presence of NSP2 nor of its NTPase activity on the ATPase activity of NSP5 *in vitro*.

Attempts to detect critical aminoacids on either of the *in vitro* activities of NSP5, its phosphorylation, hyperphosphorylation in the presence of NSP2 or its ATPase activity, were performed using molecular dynamics and docking on a putative structure of NSP5. After performing site directed mutagenesis and analyzing the mutant activities no critical aminoacids for these activities were detected using the present model.

NSP2 and NSP5 co-localize in the viroplasms and in the absence of either of them, the other protein has a dispersed cytoplasmic localization suggesting that NSP2 and NSP5 might interact. At the beginning of this thesis the question of interaction between both proteins was raised. Interestingly, using the methodology of ultracentrifugation in glycerol gradients, a direct interaction was not detected, in agreement with others results (data not shown). Thus, although both proteins co localize, it was not possible to detect an interaction *in vitro*. Yet, both proteins might have a transient interaction, undetectable with the methodologies applied, and thus no determination about the nature of such a possible interaction is made.

An analysis of sequences from different strains of rotaviruses did not shed light on a clear analogy between changes in NSP2 sequences and changes in NSP5 sequences

in the same strain. In resent experiments, the RNA binding of NSP2 was affected through site directed mutagenesis. These mutants were not able to co-localize with NSP5 and form viroplasms (personal communication). Thus, the interaction between NSP2 and NSP5 was first driven by an interaction with RNA. In light of these results that suggest the interaction NSP2-NSP5 is mediated by RNA the lack of correlation between the changes in sequences of both proteins could be explained since their co-localization seems to be due to a protein-RNA-protein interaction and might not a be the result of a protein-protein interaction.

In this context another question is raised. According to co-transfection experiments recently performed NSP2 from group C is not able to form viroplasms like structures (VLPs) in the presence of NSP5 of group A (Dr. J. Patton, Dr. Z. Taraporewala and Dr. R. Vasquez del Carpio- personal communication), suggesting there is a specific recognition between the proteins of different groups. Why does NSP2 of group C doesn't interact with NSP5 of group A, even though both NSP2 proteins are structurally similar and able to interact with ssRNA? What other factors besides RNA binding are important in this interaction? It is still interesting to suggest there is a major importance in the interaction NSP2-NSP5 after it is mediated by ssRNA and thus many questions are raised regarding the nature of such interaction.

"The modern theory of evolution does not require gradual change. [...] Eldredge and I believe that speciation is responsible for almost all evolutionary change. [...] Thus, phyletic transformation in large populations should be very rare—as the fossil record proclaims. But small, peripherally isolated groups are cut off from their parental stock." (Gould, 1980). In order to be maintained as members of an existing group, the new

generation members have to keep enough features similar to the group. Thus, newly synthesized rotaviruses must keep most of the features as the original particle in order to still be included in this group. Although individuals keep features identical, there is variety in a group, also driven by selective pressure, for example during cellular infection. The main problem that remains in the study of rotaviruses as well as the other members of the family *Reoviridae* is the lack of reverse genetics. Thus, it is still impossible to alter the identity of rotaviruses inserting a foreign RNA molecule to a viral particle. As just mentioned, NSP2 from group C cannot complement NSP2 from group A and form VLPs during transfection. Moreover, according to Silvestri *et al.* (2004) the synthesis of the newly (+)RNAs might occur in the viroplasm protected from siRNA degradation. Thus, the viroplasms, sites of replication and encapsidation, might also function as identity keeper of the virus, regulating the uniqueness of the source of (+)RNAs to be encapsidated.

Considering the results indicating NSP5 has an ATPase activity, a comparison between both enzymatic activities, the autophosphorylation of the protein *in vitro* and its ATPase activity appears relevant.

While NSP2 has been reported as important in inducing an increase of phosphorylation of NSP5 *in vivo* and *in vitro*, there was no apparent effect of NSP2 on the ATPase activity of NSP5 suggesting some independence between both activities.

Recently, the hyperphosphorylation of NSP5 *in vivo* was proposed to be mediated by the initial phosphorylation of a serine residue (Eichwald *et al.*, 2004). Although this data was contrasted in a recent publication and thus is controversial, the importance of this residue in the context of the ATPase activity as well as the

phosphorylation *in vitro* was analyzed. Site directed mutagenesis was used to substitute this residue, serine-67, for an alanine or alternatively for an aspartic acid simulating a phosphorylation as previously described (Eichwald *et al.*, 2004). When compared to *wt* NSP5, the mutations on serine-67 did not show any effect on the level of autophosphorylation *in vitro* suggesting a separation between the autophosphorylation *in vitro* and hyperphosphorylation *in vivo* according to this model. Although serine-67 mutants might have a different behavior regarding the hyperphosphorylation of the protein *in vivo* compared to *wt* NSP5 (Eichwald *et al.*, 2004), when mutated either to an alanine, non habilitating it for a phosphorylation, or to an aspartic acid, mimicking a phosphorylation, NSP5 *wt* and mutants behaved similarly regarding the hydrolysis of ATP. Thus, neither the ATPase activity of NSP5 reported here nor its autophosphorylation and phosphorylation induced by NSP2 *in vitro* are related to the serine-67. This also suggests the phosphorylation and hyperphosphorylation *in vivo* can be separated functionally form the ATPase and autophosphorylation activity of NSP5 *in vitro*.

NSP2 was once believed to have an autokinase activity since it seemed to be autophosphorylated when incubated *in vitro* with [$\gamma$-$^{32}$P] ATP. Later studies demonstrated the phosphorylated NSP2 was the result of the formation of an intermediate of reaction during the hydrolysis of an NTP by NSP2 (Vasquez del Carpio *et al.*, 2004). Since here an ATPase activity have been characterized and NSP5 also has been reported as an autokinase, it cannot be discarded that there is a correlation between the ATPase activity of NSP5 and the autophosphorylation seen *in vitro*. This assumption

is also supported by the fact the *in vitro* phosphorylated aminoacid of NSP5 are either an histidine, a lysine or an arginine, suggesting it corresponds to a reaction intermediate. Although analyzed utilizing different methodologies and comparing different parameters, we were not able to determine whether NSP5 enzymatic activities, the ATPase activity and the autophosphorylation and phosphorylation *in vitro*, are related, particularly since site directed mutagenesis was rarely able to affect either of both activities.

It has been unclear for long whether there is any relation between the posttranslational modifications of NSP5 and its functions, as an essential protein in viroplasm formation or other functions.

NSP5 has been for long characterized as a non structural protein that is essential in viroplasm formation and undergoes several posttranslational modifications (Afrikanova *et al.*, 1996; Blackhall *et al.*, 1998; Eichwald *et al.*, 2002; Estes, 2001; Gonzalez and Burrone, 1991; Poncet *et al.*, 1997). The role of NSP5 in viroplasm formation as well as results from NSP5 silencing assays show the relevance of this protein as an essential protein in the virus cycle (Campagna *et al.*, 2005; Lopez *et al.*, 2005b; Vascotto *et al.*, 2004). Yet, it has been unclear for long whether there is any relation between the posttranslational modifications of NSP5 and its functions, whether as a structural protein in the viroplasm formation or any other function still unknown. So far, only one report suggests a direct a relationship between a change in phosphorylation pattern of NSP5 and an effect in the virus replication cycle, thus suggesting a correlation between the replication cycle of the virus and the posttranslational modification (Chnaiderman *et al.*, 2002). It is still unclear whether the phosphorylation of NSP5 or its NAc-O-Glycosylation has effect on the function of the protein and what type of effect.

One of the major difficulties is the shortage of mutants affected in the posttranslational modifications of NSP5, that could rule out the possibility the posttranslational modifications of NSP5 are only an artifact, thus, modifications on residues of the protein in the cytoplasm but not meaningful modifications, for example resulting from modifications done on unfolded protein. A recent report about the insolubility of NSP5 when hyperphosphorylated also raised the question whether there is a role of an insoluble form of the protein in the virus replication cycle (Sen *et al.*, 2006).

Recently, the phage Φ12 of the Cystoviridae, also a dsRNA virus, was shown to have a helicase, P4 (Kainov *et al.*, 2004). The assumption that the requirement of P4 for the ejection of the nascent RNA was through its helicase activity and thus dependent on its NTPase activity, actively extruding the RNA, was recently discarded (Kainov *et al.*, 2004).

However, several NTPases have been characterized and correlated with helicases and their activity. In Herpes Simplex viruses, UL9 has both, ATPase and helicase activities and interacts with other proteins involved in the replication machinery (Marintcheva and Weller, 2001). UL84 of Human Cytomegalovirus has a UTPase activity as well as being phosphorylated and was proposed as a member of the DNA replication system of the virus (Colletti *et al.*, 2004). T antigen of SV40 was shown to share domains with ATPases as well as having helicase activity (Li *et al.*, 2003).

As was previously reported, NSP2 has an NTPase activity as well as a dsRNA destabilization activity (Taraporewala and Patton, 2001), independent of its NTPase activity. Since NSP2 was recently characterized as an RTPase (Dr. J. Patton, Dr. Z. Taraporewala and Dr. R. Vasquez del Caprio- personal communication), an important

question that remains unanswered is whether NSP5 has an energetic role in a possible helicase function of NSP2, similarly to other examples of ATPases related to helicases (Bisaillon *et al.*, 1997; Li *et al.*, 2003; Marintcheva and Weller, 2001). One of the possibilities is that NSP5 is the protein in charge of an energetic activity that could be related to the replication of the RNA (for example 'giving' an energetic push to NSP2) helping the unwinding of dsRNA secondary structures during the process of replication. Thus, the ATPase activity of NSP5 could be related to a motor function of the viral proteins in the process of unwinding the secondary structures of RNA during an encapsidation process, probably performed by NSP2. This could also explain the positive effect of dsRNA on the ATPase activity of NSP5.

In other instances NSP5 might have a role in other processes regarding possible interactions between cellular factors with the viroplasms, viral proteins and the viral replication machinery. Another possible role of NSP5 could be in cellular transcription or in movement of viral or cellular proteins to or from the viroplasms. Since several cellular pathways of signal transduction are related to proteins with nucleotide hydrolytic activities (for example G proteins) as well as phosphorylated proteins, another possible role of NSP5 could be related to signal transduction pathways correlated to infection or innate immunity responses.

## PERSPECTIVES

During this research several new questions rose regarding NSP5, its enzymatic activities, an ATPase activity, an autophosphorylation activity, and the relationship of those to the posttranslational modifications of NSP5. An important advance in this subject will be the silencing of these activities through mutagenesis and the analysis of its effects on viroplasm formation, phosphorylation of the protein in co transfection experiments among others.

One of the interesting findings of this research, not discussed here in detail, is the ability of NSP5 not only to hydrolyze ATP, being an ATPase, but also to function as an RTPase. Thus, similar to the example in reoviruses, rotaviruses have 2 proteins with both activities, NTPases and RTPases. Yet, the specificity of NSP5 to hydrolyze ATP and not other NTPs in an efficient way indicates there might be other major differences between the roles of the proteins in both viruses.

During the analysis of several gene 11 sequences we discovered an interesting finding regarding the second ORF of this gene, NSP6. While sequences from samples recovered from patients maintained the ORF of NSP6, some sequences recovered from laboratory strains lost the ORF of NSP6. This could indicate a selective pressure that was lost in cell culture, probably regarded to immune responses in patients, not present in cell culture, which caused the loss of NSP6 ORF in cell culture. According to this data, NSP6 could be related to the suppression or evasion of immune responses of the host during infection.

## CONCLUSIONS

- Bacterial purified NSP5 was found to possess the ability to hydrolyze ATP *in vitro*.
- A characterization of the conditions of the reaction of hydrolysis allowed the determination of the most convenient conditions for the hydrolysis assays. NSP5 was found to hydrolyze ATP in the β-γ phosphodiester bond in the presence of $Mg^{+2}$. Other nucleotides where not hydrolyzed to the same extent as ATP by NSP5.
- Neither sequence specific nor random sequence ssRNA had an effect on the ATPase hydrolysis of NSP5.
- Double stranded RNA had a mild positive effect on the hydrolysis of ATP when incubated with NSP5.
- The ATPase activity of NSP5 was not affected neither by the presence of NSP2 nor by its NTPase activity.
- Mutation on serine 67 of NSP5 did not have an effect neither on the phosphorylation of the protein *in vitro* nor on the ATPase activity of NSP5.
- Several evidences suggest the phosphorylation and hyperphosphorylation *in vivo* is not directly related to the ATP hydrolysis and the phosphorylation of NSP5 *in vitro*.

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"הגוזלים שלי עזבו את הקן

פרשו כנפיים ועפו

ואני ציפור זקנה נשארתי בקן

מקווה מאוד שהכל יהיה בסדר

תמיד ידעתי שיבוא היום

שבו צריך להיפרד

אבל עכשיו זה ככה בא לי פתאום

אז מה הפלא שאני קצת דואג

עוף גוזל

חתוך את השמיים

טוס לאן שבא לך

רק אל תשכח

יש בשר בשמיים

גור לך."

אריק איינשטיין

אז מה גיליתי במהלך המסע הזה בשם דוקטורת?

גיליתי שהדבר הייחיד והמקסים ביותר שניתן להיות בחיינו הוא מלוים וצופים בחיי אלה שאנו אוהבים,

ולפעמים נופל בחיקנו גם להראות להם שניה של קסם ויופי. גם כשהתוצאה תהיה לאבד את מקומנו כמלוים,

הרי את העצב הזה ממתיקים בצפיה באותה טיסה אל קסם חדש.

תודה לכל מלוי ודרך צלחה לציפורי האהובות,

תמרה