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Functional Analyses of Mammalian Reovirus Nonstructural Protein μNS*

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Abstract: Genome replication of reovirus occurs in cytoplasmic inclusion bodies called viral factories or viroplasms. The viral nonstructural protein μNS, encoded by genome segment M3, is not a component of mature virions, but is expressed to high levels in infected cells and is concentrated in the infected cell factory matrix. Recent studies have demonstrated that μNS plays a central role in forming the matrix of these structures, as well as in recruiting other components to them for putative roles in genome replication and particle assembly.

Key words: dsRNA virus, Mammalian orthoreoviruses, Nonstructural protein μNS

The nonenveloped mammalian orthoreoviruses (MRV), as a prototype member of reovirus in the family of *Reoviridae*, has a genome composed of 10 double-stranded RNA (dsRNA) that encode 11 proteins. The virion has two concentric protein capsids that form icosahedrons about 75nm in diameter. Of the 11 proteins, the eight viral proteins in the mature virion (also known as structural proteins) have particle-based functions that are critical to initiation of infection and synthesis of the capped viral mRNAs. The structural proteins are consisted of core(λ1, λ2, λ3, σ2, μ2) and outer capsid shell (μ1, σ1, σ3), of which the σ1，λ2，λ3 and μ2 proteins are locate around the icosahedral fivefold axes from the outer to the inner capsid shell**.** The remaining 4 proteins, called nonstructural proteins, are not presented in the mature virion, but are expressed in reovirus infected cells during the virus replication cycle (9, 13, 14, 23, 26).

The transcription and replication of MRV starts by the internment of the core particle in the target cytoplasm. Initially, [+]RNAs transcription occurs in the intermediate subvirion particle (ISVP) or core that contain viral genomic dsRNA. As soon as the new synthesized RNA has been transcribed, it is released into the cytoplasm from the channel of λ2 at the five-fold axes of icosahedral. Once they have been released, they can function as mRNA for translation of new viral proteins or for packaging into new viral particles with their complement [-]RNAs (10, 13, 15, 29).

When nascent virion protein components emerge, they are found to be assembled in unique cellular constructions called viral factories (21) or viral inclusion bodies (VIB) (24). The factories have a

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peculiarly dense consistency that is easy to distinguish from the adjacent cytoplasm and causes them to appear highly refractive by phase-contrast microscopy; they are quite small in the early stages of infection and then grow larger and move toward the nucleus as infection proceeds. Later in infection, the inclusion usually anchored to and spreads along stabilized microtubules in many MRV species, but not in all strains (18, 21). Reovirus factories were determined to contain fully and partially assembled viral particles, viral proteins, dsRNA and microtubules, but membranebound structures or ribosomes are not found (22, 30).

μNS is determined to play an important role in the formation of viral factory structures. Immunostaining and immunofluorescence (IF) microscopy revealed that μNS can be found to be concentrated in viral factories(21, 22, 25). The features of one or more viral proteins that would make μNS capable of forming such a matrix are not well understood but might involve a variety of different types of intersubunit interactions, as well as interactions with cellular factors. There have been several recent studies which have elucidated the role of μNS in the formation of the matrix of viral factories in virus genome replication and particle assembly by clarifying the μNS protein function and its interaction with other proteins in the virus replication cycle.

GENOME STRUCTURE OF PROTEIN μNS

The protein μNS, which coded by the Reovirus M3 segment, consists of 721 amino acids (about 80kDa). According its predicted secondary structure (Fig.1), there are two alpha regions between the 100-340 and 400-680 amino acid residues which are separated by beta regions at positions 350 to 400 aa. In particular, the μNS protein has two predicted coiled-coil segments in the carboxyl-terminal one-third of its sequence around the 550 and 650 aa region, which may be important in forming inclusions in infected cells. Significantly, there is also a negative region around the 1-40 aa residues of μNS, which is considered to have a function which is important in the interaction with structural protein μ2. Additionally, there may be another form of μNS found in infected cells, called as μNSC, which lacks about 5 kDa from the N-terminus (27). This protein is believed to be a translation product of the M3 segment from second

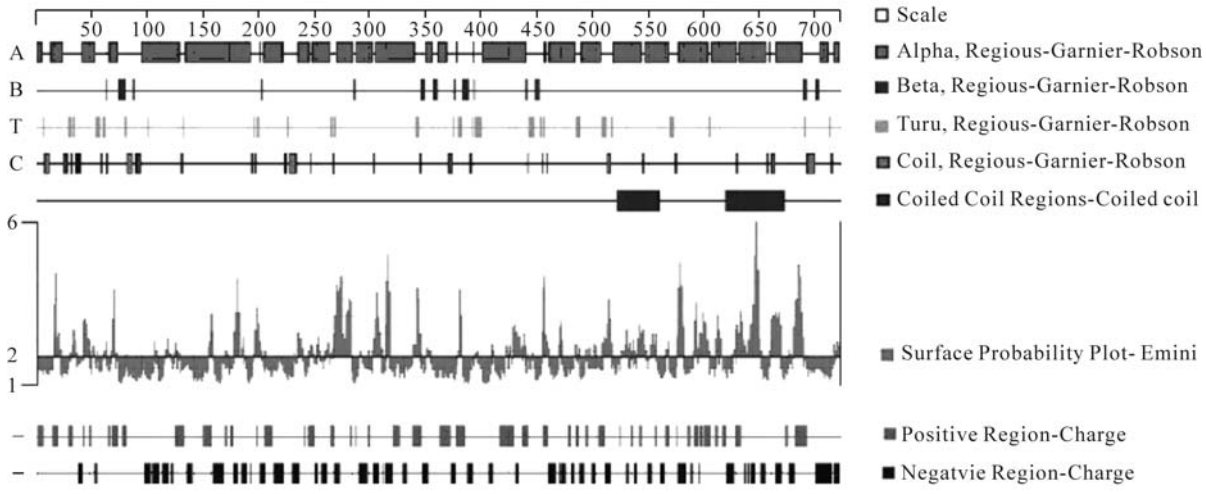

Fig.1. Sequence analysis of the secondary structure of μNS.

AUG at Met41 of the μNS sequence, but is not a digested product of μNS (17, 27). The function of it has not been determined because it is neither essential nor sufficient to infection of MRV (12). Both μNS and μNSC are present in the infected cell at a μNS: μNSC ratio of 1:1 to 4:1 (27).

STRUCTURE BASED FUNCTION OF μNS

Experiment evidences have shown that the N-terminal 40 residues of μNS are involved in associations with at least two other reovirus proteins: microtubule-binding protein μ2 and ssRNAbinding protein σNS (6, 17). The domain from N-terminal 1-41 residues determines the colocalization with minor core structural protein μ2. It is reported that protein μ2 is related to microtubules-bonding in reovirus infected cell and it can be induced into inclusion by μNS (6, 16). Molecular biology experiment indicated that deletion of μNS 1-41aa residues did not colocalize with μ2, but affected the morphology of inclusion that is formed by changing it into a globular structure in the T1L stain, forming a filamentous-like inclusion in common (6). Moreover, the specific residues necessary or sufficient for these activities have been shown to be separable. Residues 1 to 41 of μNS are sufficient for association with μ2 (5), but only some portion of residues 14 to 40 of μNS is necessary for this activity (17). In contrast, some portion of residues 1 to 13 of μNS is necessary for association with σNS (17), and residues 1 to 12 of μNS may be sufficient for this activity (7).

As indicated in the prediction of μNS secondary structure, the carboxyl-terminal region from 550 to 605 aa is critical for the formation of viral factory matrix, and the predicted coin-coiled structure is notably conserved at the His570 and Cys572 sits. To confirm if the C-terminal region is absolutely necessary in forming inclusions in virus infected cells, a series of μNS recombinant deletion constructs, being involved in the investigation of function, have been established and analyzed. Studies demonstrated that the characteristics of viral inclusion formation is particularly dependent on two predicted coiled-coil C-terminal regions of μNS, and the C-terminal region from residue 471 to 721 is also required for inclusion formation (8).

Further detailed investigations have determined that mutation of the two conservative residues, either His-570 or Cys572, would result in loss of ability to form inclusions (8, 18). Furthermore, the minimal essential region for inclusion formation has also been mapped by GFP tagged fusion proteins, which have stabilized expression in transfected cells (7, 8). Aside from the identified N-terminal and C-terminal playing a dominant role in viral factory formation, the large central region spanning from residue 42-560 may also be involved in the interaction with core proteins λ1, λ2, and σ2 and whole core particles (4, 5). In addition, a recent study indicates that residues 42-172 also play a role in shape formation of the inclusion (8), hinting there may be an association with different domains of the protein. However, the exact interaction of μNS with different domains stills needs to be clarified with further studies. The identified functional regions of μNS are summarized in Fig.2.

INTERACTION OF μNS WITH OTHER PROTEINS OR RNA

The functional domains and the role of nonstructural proteins being in virus infection and replication have been also been demonstrated. In fact, many

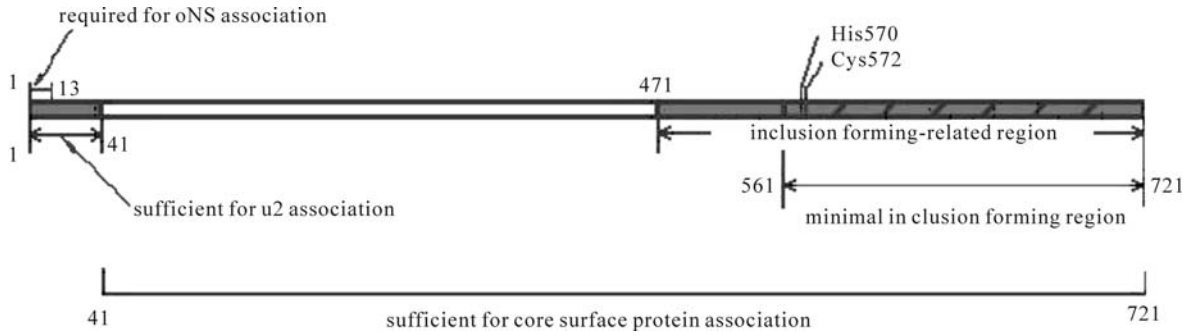

Fig.2. Function regions of μNS.

proteins, such as the microtubule-binding core protein μ2 and the nonstructural protein σNS as well as core frame proteins λ1, λ2, and σ2, are all recruited to protein-protein interactions in viroplasm formation during virus infection and particle assembly (5, 7, 17). Furthermore, there is also much indirect evidence which indicate that μNS may also possess ssRNA-binding activity, suggesting that μNS may associate with plus-strand RNA transcription during virus infection and replication despite the identification of the ssRNA-binding activity of σNS (4, 11, 12, 17).

In an earlier study, antibodies to μNS coimmuno-precipitated the viral plus strand RNA transcripts soon after the transcripts were synthesized in infected cells (1). So there is reason to believe that μNS may induce viral RNA to viral factories during viral infection, even though this may not be a direct interaction. When μNS is isolated from infected cells, it would associate with different types of viral particles, from which it can also be concluded to be a sign that the μNS has a function for assembling different viral proteins into virion particles (31).

Moreover, studies have shown that μNS has interactions with other viral proteins, including all five core proteins (λ1, λ2, λ3, μ2, and σ2) and nonstructural protein σNS, all of which can associate with μNS in these structures, suggesting that μNS is important in viron assembly and formation of viral factories (1, 4, 6, 7). Evidence has shown that each core protein (λ1，λ2，λ3，μ2 ,σ2) can be separately recruited into inclusions when co-expressed with μNS (7, 19, 20). Though the expression of core proteins from either vaccinia or baculovirus vectors allows the assembly of core-like particles in the absence of μNS coexpression (28), the vaccinia- or baculovirus-gene- rated core-like particles lack genomic RNA. So it can at least be hypothesized that the core assembly which associate with genomic RNA occurs within the viral factories (7).

σNS also performs a role in viral protein assembly, investigations have shown that viral RNA-protein complexes contain σNS and this complex can also recruit other viral proteins. However, σNS alone is not sufficient for formation of viral factories (3, 19), which indicates σNS may just have an assistant role in this process. This interaction may block the transport of infected cells, which may in turn demolish the cell metabolism and counteract the cell response toward viral infection. The anchor points may then act as seed sites of viral factories. But in the T3D strain, because μ2 has lost the ability to anchor to the microtubule, the anchor of μNS/μ2 toward the microtubule should be only an assistant process during infection while μNS/μ2 interaction must play another important role

in viral core assembly. Two proteins, μ2 and σNS, associate with the same region of μNS, which may have related roles in the viral infection process. Specifically, μ2 and σNS both associate with N-terminal 1-41 residues of μNS (7). It is possible that they have a competition relationship each other with μNS interaction. Or, the adjacent sites on μNS have a key role in recruiting viral genome dsRNA into the viral core, as μ2 are localized in the fivefold axes of the core where the RNA synthetase and viral dsRNA channel are localized while σNS is a RNA bonding protein.

The 41-721 residues of μNS are associated with the formation of viral factories and recruitment of viral core proteins (λ1, λ2, σ2). Two self interaction sites have been localized in this region: (i) a putative zinc-hook motif at residues His570 and Cys572 (ii) the extreme C terminus, which can be localized to the last two residues Asp720 and Leu721 (8, 18). The two regions are thought to be involved in μNS-μNS interactions and constructing the large three-dimensional matrix of viral factories and these interactions may lead to formation of the basic framework of viral factories in infected cells (8, 18). Meanwhile, μNS core proteins (λ1, λ2, σ2) are also identified as associating with this region (41-721), λ2 localizing in the fivefold axes and σ2/λ1 constructing the inner capsid of the virus. In such a case, there are two possibilities which may involve: (i) μNS performs as a bracket to assemble λ1，λ2, σ2 together through different sites or (ii) core proteins λ1，λ2, σ2 are first assembled to form the icosahedral of the fivefold axes by themselves and they then associate with μNS which results in their assembly as the viral core.

Interestingly, the two forms of μNS, μNS and NSC, and proportion of expression between 1:1 to 4:1 detected in infected cell (27), may be important in formation of inclusions and the viron assembly process. Neither μ2 nor σNS in transfected cells can associate with μNSC while they have interact with μNS. The two forms both have the ability in mediating inclusion. As a result, μNS and μNSC may have different effects in the viral life cycle according to their different capability.

From the evidence described above, it is possible to construct a flow chart to describe the multiple events to show how μNS is involved in cell factory formation and association with other proteins in virus replication (Fig.3). There are 3 steps for μNS to interact with other proteins during genome replication: (i) viral proteins synthesis outside the factories in the viral infection process. (ii) μNS forms inclusions in the cytoplasm and recruits core proteins into them to form viral factories. (iii) virus proteins assemble in viral factories to product new virons.

CONCLUSION AND PERSPECTIVE

In the process of inclusion formation, μNS may have two kinds of interaction; contact with itself and association with other viral proteins. The self-contact can form the framework of viral factories while association with other proteins introductions core proteins into the inclusions. The ability of μNS to switch between these two functions is possibly attributed to its concentration in the cytoplasm; at normal concentrations it may preferentially attach to core proteins and when in the vicinity of nearby inclusions where concentration of μNS reaches a high level it detaches from other proteins and interacts with itself. In this manner, core proteins can be transported

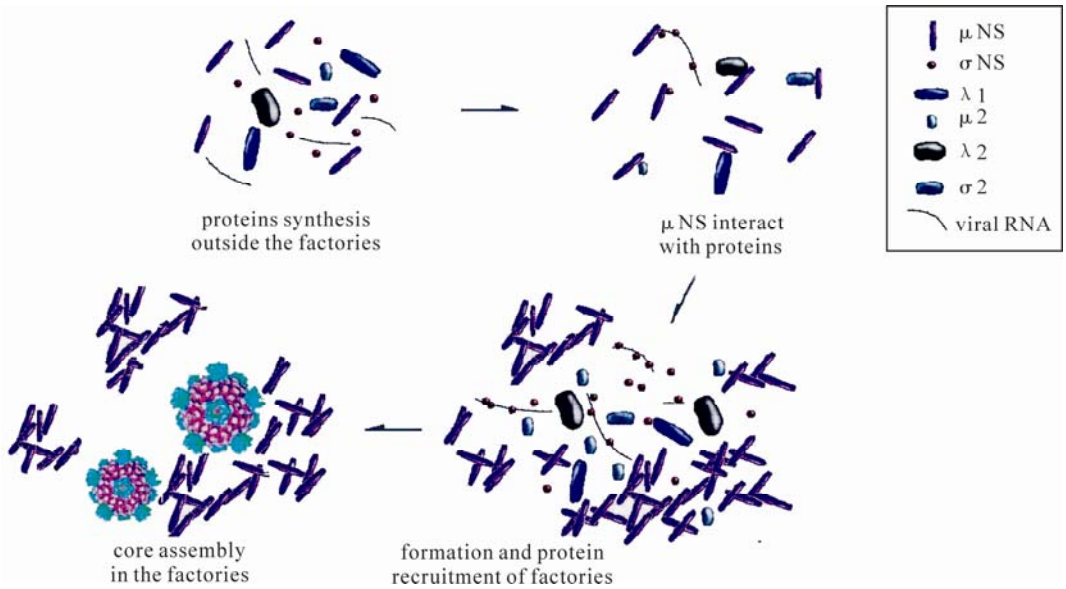

Fig.3. Schematic of μNS association with other proteins in forming viral factories.

into inclusions and be concentrated in preparation for further assembly.

There have been many studies investigating the function of μNS in the viral infection process. Given its ability to interact with RNA-binding protein σNS, μNS likely plays an important role in viral RNA recruitment into the virion to form core particles. Based on the current reports focused on the function of μNS in viral growth(6, 8, 18), a possible model of μNS role in viral core assembly in the factory is outlined Fig. 4. The possible core assembly hypothesis associated with μNS and other viral proteins in viral factory is as follows: when core proteins construct the five axes of the virion, μNS transports RNA via σNS to localize in the five-fold axes, where μNS attaches to μ2 so that the viral RNA can contact with the μ2/λ3 complex (acting as RNA synthetase). Afterwards, μNS is detached and the original assembled complex is formed. These five-fold complexes then come into contact each other and finally form the core particle.

The μNS protein plays a central role in recruiting viral components to the factories. These proteins may

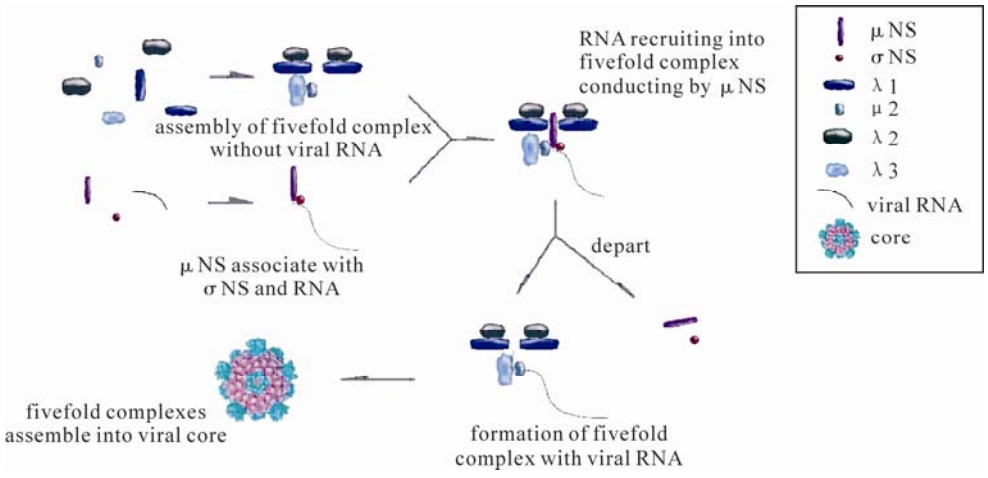

Fig.4. Model of core assembly in viroplasm.

be individually or cooperatively involved in RNA assortment, minus-strand synthesis, and core assembly. But the questions remain as to how the μNS forms factory-like inclusions, and whether there is a multifunctional role of μNS in regulating the transcriptional activity of the core particle after outer capsid assembly. Further study on formation of μNS in clusions will provide a useful tool to study protein-protein associations inside cells during virus infection.

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