The nucleoprotein of Marburg virus is phosphorylated

Stephan Becker, Sabine Huppertz, Hans-Dieter Klenk and Heinz Feldmann*†

Institut für Virologie, Robert-Koch-Straße 17, 35037 Marburg, Germany

The nucleoprotein (NP) of Marburg virus (MBG), a filovirus, is encoded by the gene closest to the 3' end of the non-segmented negative-strand RNA genome. Sequence comparison has indicated that NP is the functional equivalent to the nucleoproteins of paramyxoviruses and rhabdoviruses. Expression of recombinant NP in two eukaryotic systems using vaccinia virus and baculovirus (vectors pSC11 and pAcYM1, respectively) and analysis of MBG-specific proteins have demonstrated that the NP of MBG is phosphorylated. The NP appeared in two forms differing in Mr by about 2K (94K and 92K respectively). Dephosphorylation clearly demonstrated that the 94K form is phosphorylated whereas the 92K form is unphosphorylated. In virion particles NP was exclusively present in the phosphorylated form. These findings suggest that only the phosphorylated NP can form nucleocapsid complexes and interact with the genomic RNA.

Introduction

The family Filoviridae accommodates Marburg virus (MBG), the prototype of the family, Ebola virus (EBO) and Reston virus (RES) (Kiley et al., 1982; Murphy et al., 1990). Filoviruses are classified in the order Mononegavirales (Pringle, 1991) which also contains the non-segmented negative-strand (NNS) RNA virus families Paramyxoviridae and Rhabdoviridae. MBG and EBO are extremely pathogenic for humans and non-human primates, causing a severe haemorrhagic disease with high mortality rates.

MBG particles consist of at least seven structural proteins: the L protein (Mühlberger et al., 1992), the glycoprotein (GP) (Will et al., 1993), the nucleoprotein (NP) (94K) (Sanchez et al., 1992), and four viral structural proteins (VP) with Mr's of 38K (VP40), 32K (VP35), 28K (VP30) and 24K (VP24) (Kiley et al., 1988). The corresponding genes are located on the NNS RNA genome in the following linear order: 3' untranslated region-NP-VP35-VP40-GP-VP30-VP24-L 5' untranslated region (Feldmann et al., 1992).

The mature NP in MBG particles has an Mr of 94K and is the major component of the viral nucleocapsid. It represents approximately 27% of the virion protein mass (Kiley et al., 1988). The nucleic acid sequence of the NP gene has been recently elucidated (Sanchez et al., 1992).

Comparison of the deduced amino acid sequence showed a high degree of homology to the N-terminal 400 amino acids of the EBO NP. A small region in the middle of the NP sequence was found to contain a significant homology with the nucleoproteins of paramyxoviruses and to a lesser extent with those of rhabdoviruses (Barr et al., 1991; Sanchez et al., 1992).

Little is known about the mode of transcription and replication of filoviruses and their unusually high pathogenicity. Detailed knowledge of the functions of the various MBG proteins, their co- and post-translational modifications, and their interactions with virion or cellular proteins or nucleic acids would create a foundation for further investigations. Studies on paramyxoviruses and rhabdoviruses have shown that the nucleoprotein plays an important role in the transcription and replication of these viruses. The nucleoprotein encapsidates the template RNA which is the only form that is transcribed. Newly synthesized nucleoprotein seems to mediate the formation of plus-sense ribonucleoprotein complexes which are the template for synthesis of progeny minus-sense RNA (for reviews see Wagner, 1987; Kingsbury, 1991; Banerjee & Barik, 1992). In this report we present data on the expression of MBG NP in eukaryotic and prokaryotic systems and show that the mature NP is phosphorylated.

Methods

Viruses and cell lines. The Musoke strain of MBG isolated in 1980 in Kenya (Smith et al., 1982) and the strain WR of vaccinia virus were propagated in E6 cells, a cloned cell line of Vero cells (ATCC CRL...
Propagation and labelling of virus. E6 cells were infected with MBG at an m.o.i. of 0.01. Purification of viral particles and preparation of viral antigen were performed as described previously (Mühlberger et al., 1992). In TNE (0.01 M-Tris-HCl pH 7.4, 0.15 M-NaCl, 2 mM-EDTA) containing 1% SDS. When viral proteins were to be labelled, 20 laCi/ml [35S]methionine or [32P]orthophosphate (Amersham) was added 5 days post-infection (p.i.), and labelled virus was harvested 24 h thereafter. For viral genomic RNA isolation, a protocol described in detail by Mühlberger et al. (1992) was used.

SFVL cells were infected with AcMNPV or recombinant AcMNPV (BVNP) at an m.o.i. of 1. Virus growth (27 °C) and purification were carried out as described elsewhere (Kuroda et al., 1986). For in vivo labelling experiments, SFVL cells were infected at an m.o.i. of 10. Forty-eight hours p.i., infected cells were starved with the appropriate nutrient-deficient medium for 2 h, labelled for 2 h by adding 100 μCi/ml [35S]methionine or [32P]orthophosphate, washed twice with PBS and lysed in RIPA buffer as described above.

Molecular cloning. The NP open reading frame (ORF) was synthesized from vRNA as described previously (Feldmann et al., 1992) using the following two sets of NP-specific oligonucleotides: (i) a primer set with a KpnI site (underlined), 5' CAGGGTACCGTGTTACATATAAAATAGAAGATATTAC 3' (mRNA sense, bases 31 to 61) and 5' CAGGGTACCGTGTTACATATAAAATAGAAGATATTAC 3' (vRNA sense, bases 2967 to 2939) and (ii) a primer set with a KpnI site (underlined), 5' CAGGGTACCGTGTTACATATAAAATAGAAGATATTAC 3' (mRNA, bases 98 to 126) and 5' CAGGGTACCGTGTTACATATAAAATAGAAGATATTAC 3' (vRNA sense, bases 2375 to 2347). The numbering is according to the genome position (for reference see EMBL Data Library, embl: MVRNPCYC; accession number Z12132). The fragment obtained by using the primer set with KpnI sites was ligated into the KpnI site of the plasmid vector pAcYM1B (Matsuura et al., 1987). The fragment obtained by using the primer set with SaI sites was ligated into the SaI site of the plasmid vectors pUEX (Bressan & Stanley, 1987) and pSC11 (Chakrabarti et al., 1985). For cloning into pSC11, the plasmid vector was altered by introducing a SaI linker into the unique SaI site of the plasmid (Fig. 1). Following cloning, the entire NP ORF was sequenced in all recombinant plasmids (pUEX-NP, pAcYM1B-NP and pSC-NP) using the chain-terminating inhibitor method (Sanger et al., 1977).

Expression of NP in Escherichia coli and production of polyclonal anti-NP sera. E. coli cells were transformed with pUEX-NP. Bacteria were grown at 30 °C and recombinant clones were selected by their ampicillin resistance. For expression studies, bacteria were grown for 5 h at 30 °C in 2YT medium containing 100 μg/ml ampicillin. Expression of the β-galactosidase–NP fusion protein (β-gal–NP) was

![Diagram](https://example.com/diagram.png)
induced by shifting the temperature to 42 °C for 2 h. Bacteria were pelleted at 3000 g for 10 min at 4 °C, washed once in TNE, and resuspended in a reducing lysis buffer (10% v/v glycerol, 0.187 M-Tris-HCl pH 8.8, 3% v/v SDS, 5% v/v 2-mercaptoethanol, 0.05% w/v bromophenol blue). Proteins were subjected to SDS-PAGE (Laemmli, 1970), and the gel was stained with 0.3 M-CuCl₂. The β-gal-NP fusion protein band was cut out and incubated in a buffer containing 250 mM-Tris-HCl pH 8.0, 1 mM EDTA for 30 min (buffer was changed three times) followed by PBS. The sample was lyophilized, ground to powder, and resuspended in PBS. The suspension was used to immunize guinea-pigs. Immunization was performed with a 1:1 (v/v) mixture of antigen and Freund's complete adjuvant (Behring). Animals were boosted six times with a 1:1 (v/v) mixture of antigen and Freund’s incomplete adjuvant (Behring). Screening for antibody production was done by immunoblotting and indirect immunofluorescence assays.

Transfection of SFVL cells and selection of recombinant AcMNVP (BVNP). Transfection of SFVL cells (2.8 x 10⁶ cells in a six-well tissue culture plate) was performed using the lipofectin precipitation technique (Felgner et al., 1987) as described by the manufacturer (Gibco BRL) with 1 μg AcMNVP wild-type DNA (Baculo Gold, Dianova) and 2 μg pAcYM1B-NP DNA. Five days post-transfection the virus titres of the supernatant fluids were determined by plaque assay as described elsewhere (Kretzschmar et al., 1992). Screening for recombinant viruses was done by dot-blot hybridization according to the method of Pen et al. (1989) using a digoxigenin-labelled NP-specific probe (digoxigenin luminescent detection kit; Boehringer Mannheim). For this, 3 x 10⁶ SFVL cells cultured in 96-well tissue culture plates were infected with 20 p.f.u. of virus. The supernatant fluids of positive wells were used in plaque assays in order to obtain purified recombinant BVNP (Kretzschmar et al., 1992).

Construction of recombinant vaccinia virus (vSCNP). Construction of pSC-NP was performed according to the method described by Chakrabarti et al., 1985). For selection of recombinant virus the human cell line TK-143 was used (Rhim et al., 1975). Briefly, 1.5 x 10⁹ TK-143 cells were infected with vaccinia virus (WR strain) at an m.o.i. of 0.01. Cells were transfected (2 h p.i.) with 2.5 μg pSC-NP DNA by the lipofectin precipitation method (Felgner et al., 1987), and 24 h post-transfection the medium was replaced with Dulbecco’s medium containing 10% fetal calf serum (FCS). Forty-eight hours post-transfection cells were scrapped into the medium, pelleted, resuspended in 0.5 ml Dulbecco’s medium without FCS, and lysed by three cycles of freezing and thawing. This suspension was used for plaque screening on TK-143 cells in the presence of 25 μg/ml bromodeoxyuridine (Mackett et al., 1986). The plasmid vector pSC11 is constructed to express two foreign proteins, β-galactosidase under the control of the vaccinia virus 11K promoter and an additional foreign gene (NP) under the control of the 7.5K promoter (Chakrabarti et al., 1985). Recombinant viruses (vSCNP), generated by homologous recombination, were plaque-purified three times on TK-143 cells using selection for β-galactosidase-positive plaques. Forty-eight hours p.i., plaque medium containing 300 μg/ml X-Gal was added, and screening for TK/β-galactosidase² plaques was done 6 to 8 h later. Positive plaques (recognized by their blue colour) were further plaque-purified three times.

Dephosphorylation. Dephosphorylation of the NP was performed according to the method of Pr6haud et al., 1990). Following in vivo labelling with [³²P]orthophosphate or [³⁵S]methionine, infected cells were lysed in a buffer containing 0.5% NP40, 20 mM-Tris-HCl and 150 mM-NaCl pH 8. One part of the cell lysate was dephosphorylated using 0.1 U/ml calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) for 30 min at 37 °C. Controls were treated in the same way except that the CIP was omitted. After dephosphorylation, cell lysates were immunoprecipitated as described elsewhere (Huber et al., 1991) using an anti-β-gal–NP guinea-pig serum (dilution 1:100) or anti-MGB guinea-pig serum (dilution 1:100) as indicated in the corresponding figures.

Immunoblot analyses. Viral proteins were separated on 10% SDS-polyacrylamide gels as described by Laemmli (1970). After electrophoresis proteins were blotted onto PVDF membranes (Millipore) by the semi-dry blot technique. Immunodetection was performed using either the polyclonal anti-β-gal–NP serum (1:300) or an anti-MGB serum raised in guinea-pigs, and a rabbit anti-guinea-pig secondary antibody coupled to horseradish peroxidase (1:500).

Indirect immunofluorescence tests. SFVL cells and E6 cells were cultured on glass coverslips and infected with AcMNPV or BVNP at an m.o.i. of 10 and vaccinia virus (WR) or vSCNP at an m.o.i. of 5 or 10, respectively. For SFVL cells, cells were rinsed with PBS 48 h p.i., fixed with 3% paraformaldehyde for 30 min, and the plasma membranes were lysed by 30 min in 2% Triton X-100 in PBS (containing calcium and magnesium). E6 cells were rinsed with PBS and fixed with cold acetone (−20 °C) for 10 min. After fixation, cells were incubated for 1 h at room temperature with different primary antibodies. For vSCNP-infected E6 cells this was an anti-MGB guinea-pig serum, diluted 1:40 in PBS, and for BVNP-infected SFVL cells an anti-β-gal–NP guinea-pig serum, diluted 1:40 in PBS. Subsequently, cells were washed three times with PBS and incubated with a fluorescein isothiocyanate-conjugated rabbit anti-guinea-pig antiseraum (Dako) diluted 1:50 in PBS, for 1 h at room temperature. Finally the cells were washed three times with PBS and examined using a fluorescence microscope (Zeiss, Axiomat).

Results

PCR amplification and cloning of the entire NP ORF

The entire ORF of the NP gene of MBG strain Musoke was synthesized from genomic RNA (vRNA) by RNA PCR using two sets of specific oligonucleotides (Fig. 1). The NP ORF starting with the AUG codon at positions 104 to 106 and terminating at positions 2189 to 2191 (UAG) is 2088 nucleotides long and encodes a protein of 695 amino acids with a predicted Mr of 77,865 (Sanchez et al., 1992). The DNA fragment synthesized using the primer pair with KpnI sites had a length of 2937 nucleotides (positions 31 to 2967) and contained a part of the 3' untranslated region of the genome (16 nucleotides), the entire NP gene including the transcription initiation and termination signals (positions 47 to 2843), the NP–VP35 intergenic region, and part of the VP35 gene (positions 2851 to 2967). This fragment was cloned into the plasmid vector pAcYM1B under the control of the polyhedrin promoter using the KpnI site of the multiple cloning site (MCS). The DNA fragment produced using the primer pair with the SalI sites had a length of 2278 nucleotides (positions 98 to 2375) and contained the last four nucleotides of the 3' non-coding region of the NP gene, the NP ORF, and 184 nucleotides of the 5' non-coding region of the NP gene (transcription signals are not included) (Sanchez et al., 1992; Feldmann et al., 1992). This fragment was cloned into the plasmid vector pUEX using the SalI site of the MCS and the
Expression of recombinant NP

Expression from the pUEX plasmid is controlled by the cI857 repressor gene, whose temperature-sensitive protein product shuts off the Pr promoter. Expression is achieved by transferring the host cells from the normal growth temperature (30 °C) to 42 °C. In this system, the NP was expressed as an insoluble fusion protein in which the fusion point was close to the C terminus of the β-galactosidase. The fusion protein had a predicted Mr of approximately 200K and migrated on SDS-polyacrylamide gels as expected. In immunoblot analysis, the fusion protein reacted specifically with an anti-MBG guinea-pig serum demonstrating that the NP ORF was expressed as a part of the fusion product (Fig. 2a). The fusion product β-gal–NP was easily separated by SDS-PAGE from other E. coli proteins owing to its high Mr. It was used in a polyacrylamide gel suspension for immunization of guinea-pigs. After six immunization
boosts the sera reacted positively in immunoblot analyses to the mature viral NP as well as to recombinant NP with titres of 1/1000 to 1/2000. These polyclonal anti-β-gal–NP sera were used at dilutions of 1:40 in indirect immunofluorescence tests, 1:100 in immunoprecipitation assays and 1:300 in immunoblot analyses.

Expression of the NP ORF in both mammalian cells (E6), by recombinant vaccinia virus (vSCNP), and insect cells (SFVL), by recombinant baculovirus (BVNP), resulted in a protein product of the expected Mr (Kiley et al., 1988; Sanchez et al., 1992) migrating in SDS–PAGE as a 94K protein and comigrating with the mature viral NP. Immunoblots with lysates of BVNP-infected SFVL and vSCNP-infected E6 cells using the monospecific anti-β-gal–NP sera confirmed the expression of MBG NP in both systems (Fig. 2b and c). Additional protein bands of lower Mr, which also reacted with the sera used for immunoblot analyses represent degradation products of the NP and were also commonly seen in preparations of MBG structural proteins (Fig. 2a and b, lanes 3). BVNP-infected SFVL cells expressed high levels of recombinant NP constituting approximately 10% of the total cellular protein mass. The protein could be visualized by Coomassie blue staining as shown in Fig. 5(b). In contrast, the level of NP expression in E6 cells did not allow detection by Coomassie blue staining.

The expression of recombinant NP could also be shown by in vivo labelling experiments in both eukaryotic systems using [35S]methionine (Fig. 3a and b and Fig. 5a). In both expression systems (vaccinia virus and baculovirus) the recombinant NP appeared as a double band in SDS–PAGE. The lower mobility band had an apparent Mr of 94K and comigrated with the in vivo [35S]methionine-labelled mature viral NP (Fig. 3a, b and Fig. 5a). The second band had a slightly increased mobility on SDS–PAGE, migrated with an Mr of approximately 92K and was not present in viral particles (Fig. 3a, b and Fig. 5a). These two forms were separated only when the amount of the protein loaded was low and could be also detected by Coomassie blue staining in the case of BVNP-infected SFVL cells (Fig. 5b). Both proteins reacted with the polyclonal anti-β-gal–NP sera. Infection of E6 cells with vSCNP resulted in coexpression of β-galactosidase due to the expression vector used for
Two different forms of NP

The two forms of the NP, which were found in cells expressing the recombinant NP, could also be detected in MBG-infected E6 cells by in vivo [35S]methionine labelling (Fig. 5c, lane 2). In order to investigate the nature of these two forms, BVNP-infected insect cells were labelled with [35S]methionine and the proteins were then dephosphorylated with CIP. Phosphatase treatment resulted in the loss of the 94K form of NP (Fig. 5a, lanes 4 and 5, see arrows) and an increase in the intensity of the 92K form. Fig. 5(b) shows a Coomassie blue-stained SDS-polyacrylamide gel of BVNP-infected SFVL cell lysates treated in the same manner as described for Fig. 5(a) and again it can be seen that the 94K form was converted into the 92K form. The heavily stained protein in lane 4 with an Mr of 69K represents the CIP. These data demonstrated that the NP exists in two forms, a phosphorylated 94K form and an unphosphorylated 92K form. However, in virion particles (Fig. 5a and b, lanes 3) the NP is present only in the phosphorylated (94K) form.

Intracellular distribution of the recombinant NP

Indirect immunofluorescence assays using an anti-MBG guinea-pig serum (E6 cells) or the anti-β-gal–NP serum (insect cells) were performed to determine the intracellular distribution of the NP expressed in mammalian and insect cells. Both recombinant NPs were exclusively generating the recombinant virus (Fig. 3a, lane 3, marked by asterisk).

Phosphorylation of the NP

Propagation of MBG in E6 cells in the presence of [32P]orthophosphate revealed two phosphorylated virus structural proteins (Fig. 4a). The major phosphorylated protein comigrated with the [35S]-labelled viral NP. The second, weakly phosphorylated, protein had an apparent Mr of 30K to 35K and could be either VP35 or VP30. This finding is in line with the observation made for EBO which demonstrated phosphorylation of NP and VP30 (Elliott et al., 1985). In vivo labelling experiments with vSCNP-infected E6 cells and BVNP-infected SFVL cells using [32P]orthophosphate confirmed the phosphorylation of the NP. In contrast to the double band that was detected by [35S]methionine labelling, a single labelled protein was immunoprecipitated which comigrated with the mature in vivo [32P]-labelled virion NP (Fig. 4b and c). CIP treatment of the immunoprecipitated [32P]orthophosphate-labelled proteins revealed loss of the label (Fig. 4c) whereas immunoblot analysis proved that the protein was not degraded by the treatment (data not shown).
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(a) (b) (c) (d) (e) (f)

Fig. 6. Localization of NP in infected cells by indirect immunofluorescence assay. Infection of cells and detection of expressed recombinant NP using an anti-MBG guinea-pig serum (dilution 1:40) (vaccinia virus) and an anti-β-gal-NP guinea-pig serum (baculovirus) were done as described in Methods. (a) Vero E6 cells, mock infection; (b) Vero E6 cells, vaccinia virus (strain WR) infection; (c) and (d) Vero E6 cells, vSCNP infection; (e) SFVL cells, AcMNPV (wild-type) infection; (f) SFVL cells, BVNP infection.

localized in the cytoplasm of the infected cells. vSCNP-infected E6 cells showed large, primarily perinuclear, cytoplasmic inclusion bodies (Fig. 6a to d) whereas SFVL cells infected with BVNP showed a uniform distribution of the NP in the cytoplasm (Fig. 6e and f).

Discussion

Sequence analysis of the genes encoding filovirus NPs and comparison with the sequences of other NNS RNA virus nucleoproteins suggests that filovirus NP seems to be the functional analogue of the nucleoproteins of paramyxoviruses and rhabdoviruses (Sanchez et al., 1992). This is further supported by the fact that filoviral NP genes are located at the extreme 3’ end of the viral genome as found with all NNS RNA virus nucleoprotein genes (Feldmann et al., 1992). As shown for all nucleoproteins, filoviral NPs are the major components of the viral ribonucleoprotein complex and are tightly bound within the complexes. However, in comparison to all other NNS RNA viruses, filoviral NPs have unusually high Mₖs. Whereas the nucleoproteins of paramyxoviruses range from 42K to 62K (Morgan, 1991) and those of rhabdoviruses from 47K to 62K (Nichol, 1993), the NP of MBG has an Mₖ of 94K and that of EBO an Mₖ of 104K (Elliott et al., 1985). This fact suggests that filoviral NPs may have an additional function compared with the nucleoproteins of paramyxoviruses and rhabdoviruses and this function may be located in the C-terminal part of the NP.

In order to characterize filovirus NPs better and to define their functional role we expressed the MBG (strain Musoke) NP in different cell systems. The vaccinia virus system was chosen because of its ability to infect Vero cells, a mammalian cell line that is commonly used for the propagation of all filoviruses and thus reflects more authentic conditions. The baculovirus system was chosen for expression of the protein in insect cells (SFVL cell line); this is known to provide large amounts of expressed foreign proteins (Luckow & Summers, 1988). The recombinant proteins were examined for their cellular localization and co- and post-translational modifications. In addition, the MBG NP was expressed as a C-terminal β-galactosidase fusion protein in E. coli for production of specific anti-NP sera as tools for diagnosis and research.

Expression of the MBG NP in both cell lines revealed a product of the expected Mₖ that comigrated on SDS-PAGE with the mature viral NP (Fig. 2 to 5). As expected, NP was expressed to higher levels in insect cells infected with the recombinant baculovirus (BVNP) (Fig. 5b) than in E6 cells infected with the recombinant vaccinia virus (vSCNP). Expression of NP by vSCNP could only be shown by immunoblot analysis or in vivo labelling (Fig. 2b and 3a). This phenomenon may be due in part to the fact that the NP ORF contains a vaccinia virus early transcription termination signal (3’ TTTTTAT 5’; Rohrmann et al., 1986) 393 nucleotides downstream of the AUG initiation codon (Sanchez et al., 1992).

In vivo labelling of virion particles using [³²P]orthophosphate in combination with CIP treatment has clearly demonstrated that the mature MBG NP is phosphorylated (Fig. 4). Phosphorylation is not a unique feature of filoviral NPs. Among NNS RNA viruses the
nucleoproteins of rabies virus (Sokol & Clark, 1973), Sendai virus (Lamb & Choppin, 1977) and measles virus (Norby & Oxman, 1990) are phosphorylated.

Phosphorylation of NP was also demonstrated in both expression systems investigated (Fig. 4). This indicates that phosphorylation of MBG NP could be mediated in part by cellular kinases. Acceptors for phosphorylation can be either serine, threonine or tyrosine residues depending on the particular protein kinase (for review see Leader & Katan, 1988). These amino acid residues are found throughout the MBG NP. Previous studies have shown that different phosphorylated residues on a single protein result from the action of different protein kinases. This was shown for glycogen synthetase (Cohen, 1985) and the phosphorylation of the P protein of vesicular stomatitis virus which is carried out by both cellular kinases and the kinase activities of the viral L protein (Banerjee & Barik, 1992). Whether phosphorylation of NP during MBG infection is performed in part by the viral L protein is a subject of current investigations. ATP-binding site motifs have been identified in the MBG L protein sequence indicating the possibility of a kinase function for this large protein (Mühlberger et al., 1992). For Sendai virus, Einberger et al. (1990) have demonstrated a kinase activity of the L protein by in vitro phosphorylation of NP and P protein. In the case of the recombinant MBG NP one should also consider the possibility of kinase activities related to an infection with baculovirus or vaccinia virus. Therefore our studies do not necessarily allow the conclusion that phosphorylation of recombinant NP is due to the same enzymatic activities and the use of identical acceptors for phosphorylation as in MBG-infected cells.

Expression of the NP ORF in both systems as well as in the MBG-infected E6 cells revealed the synthesis of a larger phosphorylated and a smaller unphosphorylated form of the protein (difference in $M_r$ approx. 2K). However, in virion particles only the phosphorylated (94K) form could be detected (Fig. 3 to 5). A similar phenomenon was observed when recombinant simian virus 40 large T antigen was expressed in human 293 cells. Expression resulted in two forms of the large T antigen differing in $M_r$ by 1K which was due to a conformational change related to phosphorylation of threonine residues (Grässer & König, 1992). On the other hand, the lower $M_r$ form could also be the result of a proteolytic process as shown for the major nucleocapsid protein of influenza virus. This appears, depending on the cell type, at late stages of the infection cycle in two differently migrating forms (Zhlinov & Bukrinskaya, 1981). NP$_{53}$ (53K) was shown to be the proteolytically cleaved form of NP$_{56}$ (56K). However, the fact that [$^{32}$P]orthophosphate labelling of the NP showed only the larger form to be phosphorylated and the conversion of the 94K form into the 92K form by phosphatase treatment do not support proteolytic degradation.

Indirect immunofluorescence studies demonstrated that recombinant NP is found exclusively in the cytoplasm of infected cells (Fig. 6). The immunostaining pattern which can be seen in these cells is similar to the pattern found in MBG-infected Vero cells and human endothelial cells (Becker et al., 1992; Schnittler et al., 1993). In MBG-infected cells this pattern is related to the formation of intracytoplasmic inclusion bodies which consist of viral nucleocapsids. Since viral nucleocapsids are composed of genomic RNA, NP and probably three additional viral proteins (VP35, VP30 and L) (Elliott et al., 1985; Kiley et al., 1988), our findings indicate that nucleocapsid-like structures can also be formed by spontaneous aggregation of NP molecules. Furthermore, NP molecules could also aggregate with cellular RNAs and/or proteins resulting in formation of nucleocapsid-like structures. Formation of spontaneous aggregates was also found in cells expressing recombinant nucleocapsid protein of vesicular stomatitis virus (Sprague et al., 1983) or measles virus nucleoprotein (Sphnner et al., 1991).

The exact function of phosphorylation of the nucleoproteins of NNS RNA viruses has not yet been elucidated. It is noteworthy that only the phosphorylated form of the NP is found in virion particles of MBG. This result may indicate that only the phosphorylated NP is able to interact with genomic RNA and form the nucleocapsid complex. It is known for several proteins that phosphorylation is a prerequisite for binding to RNA. The rex protein of human T lymphotropic virus type II loses its affinity for the R region of the 5' long terminal repeat after treatment with phosphatases (Green et al., 1992). Han et al. (1992) showed that phosphorylation determines the interaction between the trans-activation response (TAR) region of human immunodeficiency virus type 1 and a cellular TAR RNA stem-binding factor. In this context phosphorylation of the NP should be considered in relation to the role of this protein during the transcription and replication of MBG.

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References


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