Measles virus nucleocapsid protein expressed in insect cells assembles into nucleocapsid-like structures

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The gene encoding the major nucleocapsid, N, protein of measles virus has been inserted into a baculovirus vector under the control of the polyhedrin promoter. Insect cells infected with this recombinant baculovirus synthesize high levels of measles N protein, up to 40% of total soluble cell protein. The recombinant protein is recognized by sera from convalescent patients, vaccinees and patients with subacute sclerosing panencephalitis and thus could form the basis of a simple diagnostic assay. Nucleocapsid-like structures, similar to those found in mammalian cells infected with measles virus, can be observed in both the nucleus and cytoplasm of the infected insect cells. These have many structural features in common with nucleocapsids found in measles virus-infected cells, but are longer (up to 2 μm) and have a lower buoyant density. Measles N protein thus appears to be capable of assembling into nucleocapsid-like structures in the absence of measles virion RNA or other viral proteins.

Measles is a severe disease of childhood, responsible for more infant deaths than any other virus disease, amounting to about 2.3 million per annum, according to recent World Health Organization estimates (Henderson et al., 1988). Even in countries where the fatality rate is reduced due to improved health care and nutrition, measles causes severe life-threatening infections at a rate of greater than one in 1000 notifications. Many of these patients die or exhibit long-term neurological sequelae and some develop a fatal chronic infection of the CNS, subacute sclerosing panencephalitis (SSPE) (ter Meulen et al., 1983).

Measles virus (MV) is a morbillivirus with a single-stranded, negative-sense genome of 15893 bases, containing six genes encoding at least eight proteins (Barrett et al., 1991). The virion core contains a helical nucleocapsid composed of a single molecule of RNA, surrounded by the major nucleocapsid (N) protein, a less abundant phosphoprotein (P) and small amounts of a large (L) protein. This core is surrounded by a matrix protein and enclosed by a lipid bilayer containing the haemagglutinin (H) and fusion proteins. Thus the function of the N protein appears to be packaging of the viral nucleic acid and the formation of replication complexes, along with the P and L proteins. Little is known of the structure of the N protein although its sequence has been reported (Rozenblatt et al., 1985) and nucleocapsid complexes viewed by electron microscopy (EM) suggest that it has an elongated morphology (Waters & Bussell, 1974). The primary structure of the N protein is highly conserved, apart from a short hypervariable region at the C terminus (Cattaneo et al., 1989).

The MV N protein elicits a strong humoral immune response in patients, vaccinees and experimental animals, which is matched only by that against the H protein (Stephenson & ter Meulen, 1979). Antibodies against the N protein react well in complement fixation analyses and these have formed the basis of measles diagnosis for many years (Gershon & Krugman, 1979). Interest in the N protein has increased recently after the demonstration of its ability to stimulate the production of specific CD4+ T lymphocytes that protect against encephalitic disease in an animal model (Bankamp et al., 1991). The demonstration that protection can be elicited by an internal protein such as N indicates that cell-mediated immune (CMI) responses against internal proteins play an important part in protection against disease. This conclusion is supported by earlier observations that children with defects in their humoral immune system are more resistant to measles infection than those with defects in CMI response.

Research into the structure, function and immunogenicity of N protein has been restricted because there is
only poor growth of the virus in tissue culture, and the particles are difficult to purify. We therefore employed recombinant baculoviruses to express high levels of the MV N protein in insect cells because the synthesis of this protein does not involve transport to the lumen of the endoplasmic reticulum nor subsequent glycosylation (Ray et al., 1991).

A Bluescript KS' vector containing the N gene of the Edmonston P9 strain of MV, obtained by the techniques described by Schmid et al. (1987) was cut with SpeI and XhoI to produce a 1.7 kb fragment. The 5' untranslated region was removed to minimize possible down-regulation, and appropriate restriction sites were created using the linearized plasmid as the template in a PCR reaction employing oligonucleotides containing the pUC multiple cloning sites. Difficulties in cloning this PCR reaction product were overcome by first incorporating the PCR reaction product into the plasmid pCR™1000 used in the 'TA cloning system' (Invitrogen) to create plasmid pMV57. As PCR can cause base deletions and mutations, virtually the whole N gene was then replaced by transferring a BamHI-XbaI fragment from the original vector to create plasmid pMV58. The DNA sequence of the remaining 5' and 3' elements of the N gene in pMV58 was determined and confirmed to be correct. An additional downstream NheI site was added to allow insertion into the baculovirus transfer vector by excising the N gene from pMV58 on a PstI fragment and inserting it into a unique PstI site in the vector pSpL1180 (Pharmacia). A plasmid, pMV63, in the correct orientation to enable excision by an NheI/SpeI digest was selected. The N gene was thus excised on an NheI/SpeI fragment and transferred to the baculovirus transfer vector pJVP10Z (Vialard et al., 1990) (Fig. 1). Plasmids containing the N gene under the control of the strong polyhedrin late promoter, in the correct orientation, were selected by digesting with BamHI, and were designated pMV69. All restriction enzyme digests, agarose electrophoresis, elution from agarose gel slices, ligations, transformations and DNA purifications were performed as described elsewhere (Maniatis et al., 1989).

A recombinant baculovirus, Rbac69, containing the MV N gene driven by the polyhedrin late promoter, was selected after co-infection of Sf9 cells with pMV69 and the baculovirus AcMNPV, as described by Summers & Smith (1987). Recombinant viruses were plaque-purified three times in Sf9 cells. Because the recombinant baculoviruses contained the Escherichia coli lacZ gene driven by the p10 promoter, plaques could be rapidly identified by their blue coloration after staining with X-gal (Vialard et al., 1990). Cells infected with recombinant virus were also occlusion-negative, owing to insertional inactivation of the polyhedrin gene.

Expression of the N protein, detected by electro-}

phoretic analysis (Stephenson et al., 1977) of lysates of cells inoculated with Rbac69 by 2 days post-infection, reaches maximum levels by 3 days and remains high for at least 8 days (Fig. 2a). At 3 days post-infection most of the N protein appears in its intact form, although low levels of proteolysis can be detected. Later in infection most of the N protein is cleaved into proteins of lower relative molecular mass, in a manner similar to that observed in MV-infected mammalian cells (Rima, 1983). The observation that specific cleavage products of the N protein can be produced in insect cells as well as in mammalian cells suggests that the trypsin-like cellular proteases responsible are widespread in nearly all eukaryotic cells, although the cleavage patterns are not identical. When gels stained with Coomassie blue are scanned by densitometry, an estimate of the total amount of N protein can be made. In insect cells infected for 4 days MV N protein constitutes about 41% of the total soluble protein in the cell and is clearly seen as a major constituent of Rbac69-infected cells. The identification of the N protein, and of its cleavage products, was confirmed by Western blots (Warnes et al., 1986) with SSPE serum (Fig. 2b) or N-specific monoclonal antibodies (data not shown).

The Western blots suggested that N protein synthesized in insect cells was antigenically similar to that produced in MV-infected cells. This conclusion was supported by an ELISA, reacting the antigen with a panel of human sera (Fig. 3). All the MV-positive sera reacted well with antigen from Rbac69 cells, but not with control antigen. The MV-negative sera reacted at a low level with both N protein-containing antigen and control antigen. These results suggest that insect cells infected with Rbac69 could provide a reliable source of antigen for measles diagnosis in disease and vaccine surveillance.
The assembly of MV N protein into nucleocapsid-like structures was analysed by density-gradient centrifugation and EM. Sf9 cells were infected with Rbac69 virus, harvested after 4 days, sectioned and prepared for analysis by transmission EM as described previously (Reynolds, 1963). Nucleocapsid structures are clearly visible throughout the cytoplasm and nucleus (Fig. 4a, b). These structures were not found in cells infected with a recombinant baculovirus expressing β-galactosidase alone (Rbaclac) (data not shown) and were similar in structure to those previously observed in mammalian cells infected with MV by several other workers (for review, see Rima, 1983). Although MV is a cytoplasmic virus, with no known requirement for cellular nuclear functions, nucleocapsids are frequently found in the nucleus, especially during persistent infections (Robbins, 1983) and in neuronal cells from patients with SSPE (Dubois-Dalcq et al., 1974). Dingwall & Laskey (1991) have identified a bipartite nuclear location motif consisting of two basic amino acids, a spacer region of 10 to 15 amino acids (or in some cases up to 37 amino acids) followed by a second region where three of five amino acids are basic. A similar motif is found in the MV N protein, stretching from residues 220 to 239, consisting of two arginines, a spacer region of nine amino acids and then a region where four of the next nine amino acids are basic. This sequence is conserved in all N protein sequences published so far and is very similar to the nuclear location motif described by Dingwall & Laskey. Thus if this sequence does indeed act as a nuclear location signal it appears to operate in both vertebrate and invertebrate cells.

The physical characteristics of these nucleocapsid-like structures were analysed after partial purification on...
CsCl gradients. Infected Sf9 cells were harvested 3 days after infection, pelleted and washed in PBS. The cell pellet was resuspended in lysis buffer (10 mM-Na₃HPO₄ pH 7.0, 30 mM-NaCl, 0.25% v/v Tween 20, 10 mM-EDTA, 10 mM-EGTA and 1% NP40 v/v) and lysed by freezing and thawing four times. The lysate was clarified by centrifugation at 4 °C and 1000 g for 10 min and was loaded onto a step gradient containing 65% sucrose (5 ml) and 15% sucrose (3 ml) in STE buffer (10 mM-Tris–HCl, 100 mM-NaCl, 5 mM-EDTA, pH 7.2). The sample was centrifuged for 18 h at 100000 g and 4 °C, and material collecting at the sucrose interface was removed and dialysed against STE buffer before being centrifuged into a 15 to 30% linear CsCl gradient for 48 h at 100000 g and 4 °C. A visible band of material was removed, mixed with twice its volume of 30% (w/v) CsCl and centrifuged again under the same conditions. Nucleocapsids prepared in this way, or from crude cell lysates, exhibited a classic herringbone morphology when negatively stained and analysed by transmission EM (Fig. 4c, d). These structures had an overall diameter of 22 nm (±3 nm) with a lumen measuring 4.5 nm (±1 nm) in diameter and a pitch of 6.4 nm, very similar to nucleocapsids from cells infected with MV (Waters et al., 1962). It is interesting to note that nucleocapsid structures are readily observed in Rbac69-infected insect cells and in MV-infected mammalian cells even though extensive cleavage of the N protein has occurred. Thus cleavage of the N protein does not necessarily affect its ability to form or remain in nucleocapsid-like structures, suggesting that the conformation of the protein is maintained by strong intra- or inter-molecular non-covalent bonds within the nucleocapsid structure.
Nucleocapsid-like structures prepared from Rbac69-infected Sf9 cells had a buoyant density of 1.28 g/ml, contained N protein when examined by Western blotting, and had a size distribution between 0.5 and 2 µm, with a mean length of 1 µm (data not shown).

The observation that nucleocapsid-like structures can be formed in both mammalian and insect cells expressing MV N protein from mRNA, but not containing either genome or antigenome RNA, suggests that MV nucleocapsid assembly is different from that observed in other negative-strand viruses, such as paramyxoviruses and rhabdoviruses (Baker & Moyer, 1988; Blumberg et al., 1983). The experiments reported here, along with similar studies with recombinant vaccinia virus (Spehner et al., 1991), suggest that MV nucleocapsids assemble without the assistance of other MV gene products, and that if cellular proteins are involved they are found in both vertebrate and invertebrate cells. The nucleocapsid-like structures formed in insect cells do, however, differ in two important respects from those found in MV-infected cells. Although a wide distribution of lengths is found both in natural infections and in cells infected with recombinant baculovirus, there is a higher proportion of long nucleocapsids found in lysates from insect cells. This observation suggests that the dimensions of these structures are not limited by the size of any RNA molecules they may contain. When these nucleocapsids are further purified and their buoyant density determined by centrifugation in CsCl, the density of the capsids from cells infected with Rbac69 is only 1.28 g/ml, compared with a value of 1.31 g/ml routinely reported for capsids from MV particles (Thorne & Dermott, 1976). The lower buoyant density observed for nucleocapsid-like structures from Rbac69-infected cells is consistent with the hypothesis that they may not contain RNA, and MV N protein can self-assemble into nucleocapsid structures. However, it is possible that nucleocapsid formation depends on a nucleation event that utilizes small amounts of RNA of unspecified sequence. Thus if capsid assembly and structure in MV are not dependent on the presence of MV RNA they are significantly different from capsids in other negative-strand viruses and may explain the frequency with which measles nucleocapsid structures are observed in tissues from patients with a wide variety of diseases (for review, see ter Meulen et al., 1983).

In conclusion, we have constructed a recombinant baculovirus that produces high levels of MV N protein. This protein is found in capsid structures in both the nucleus and the cytoplasm of infected cells, consistent with the identification of a nuclear location signal in this protein. These capsids are similar in morphology to those found in MV-infected cells, except that their lower buoyant density would suggest that RNA is absent and N protein can self-assemble into nucleocapsid-like structures. These capsids are highly antigenic and could be used in an ELISA-based serological assay for measles.

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References


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