Expression of Measles Virus Nucleoprotein in Escherichia coli: Use of Deletion Mutants to Locate the Antigenic Sites

By ROBIN BUCKLAND, PASCALE GIRAUDON AND FABIAN WILD*

Immunovirologie Moléculaire et Cellulaire UM 3800-CNRS, Faculté Alexis Carrel, rue Guillaume Paradin, 69372 Lyon Cedex 08, France

(Accepted 18 October 1988)

SUMMARY

Three distinct antigenic determinants on the nucleoprotein (NP) of measles virus were localized. These epitopes were defined by three monoclonal antibodies, one of which recognized all measles virus field strains examined, whereas the other two were variable. A measles virus NP cDNA subclone representing 502 of the 525 amino acids was cloned into a bacterial expression vector plasmid (pRIT) and expressed as a Protein A–NP fusion protein in Escherichia coli. The expressed protein reacted with all three monoclonal antibodies. A series of NP gene deletions was constructed in order to locate the antigenic sites. The antigenic site identified on all measles virus strains studied, which was designated site I, was located between amino acids 122 and 150. The two variable epitopes were located at the C terminus of the protein (site II at 457 to 476; site III at 519 to 525). The structural and biological implications of these observations are discussed.

Measles virus (MV) can cause both acute and persistent infections. Although the two envelope proteins of MV have been shown to be important in immunization (de Vries et al., 1988; Norrby & Golmar, 1975; Drillien et al., 1988), the role of these and of other virus antigens in immunity is unknown. Studies with influenza A virus have now established that besides the envelope proteins, the nucleoprotein (NP) plays an important role, as it is implicated in the cytotoxic T lymphocyte response (Townsend et al., 1984).

Studies with polyclonal antibodies suggested that MV was monotypic. However, comparison of laboratory-passaged viruses using monoclonal antibodies (MAbs) revealed the occurrence of epitope variations in the haemagglutinin and matrix proteins (Sheshberadaran et al., 1983). In our studies of field strains of MV isolated in Africa, we observed antigenic variation on the NP antigen only (Giraudon et al., 1988). Further, we were able to identify a common antigenic site on all virus strains examined, and two other epitopes which varied with the isolate.

Studies with MAbs have been used in the elucidation of virus structure (Wiley et al., 1981) and in defining the heterogeneity of virus populations (Yewdell & Gerhard, 1981). Such studies result in better understanding of the correlation between the structure of proteins and their association with biological activity. Thus, MAbs to different epitopes on the haemagglutinin antigen distinguish regions that can be of use as targets for antibodies capable of passively protecting animals (Giraudon & Wild, 1985). Studies with neutralization-resistant mutants of a variety of viruses have shown single amino acid changes at the antigenic site (Wiley et al., 1981; Webster & Laver, 1980). However, this approach is limited by the nature of the biological activity, i.e. neutralization. The availability of cloned virus genes and the ability to express them in heterologous systems opens up further possibilities for the mapping of biological activities other than neutralization. Thus, the expression of subclones of the glycoprotein of Rift Valley fever virus in bacteria has permitted the definition of immunoreactive areas as short as 11 amino acids (Keegan & Collett, 1986).
In our studies on MV, we have cloned the virus genes (Gerald et al., 1986; Buckland et al., 1987) in an attempt to relate various biological activities to their protein structure. In the present study, we expressed the cloned NP cDNA and deletions of the gene in order to locate the three epitopes which we had previously identified. We showed that one antigenic site, found on all the MV strains, is located in a region towards the N terminus whereas the other two epitopes which vary with the virus strain are found at the C terminus.

Measles virus NP was produced in *Escherichia coli* using a cDNA expressed from the pRIT.2T vector (Nilsson et al., 1985). This plasmid codes for the part of Protein A (of *Staphylococcus aureus*) containing the Fc binding protein and contains the lambda Pr promoter which gives high levels of expression in prokaryotic cells. The strain of *E. coli* used (N 1876), contains the lambda ci857 temperature-sensitive repressor. This allows for temperature-inducible expression of intracellular fusion proteins in *E. coli*. Briefly, a transformant colony was transferred to 1 ml Luria broth (LB) plus 50 ng ampicillin/ml and grown overnight at 30 °C. An aliquot was diluted 100-fold in LB (plus ampicillin) and grown for 3 to 4 h until the OD$_{650}$ was 0.9. At this point an equal volume of LB at 54 °C was added to the culture and incubated at 42 °C for another 90 min, after which the culture was placed on ice for 20 min, then centrifuged.

The Protein A–MV NP fusion protein was purified from the pelleted *E. coli*. Bacteria were suspended in 25% sucrose in 0.05 M-Tris–HCl pH 8.0 and incubated with 1 mg/ml lysozyme at 4 °C for 5 min. The suspension was adjusted to 0.25 M-EDTA pH 8.0 and an equal volume of 0.1% Triton X-100 in 0.06 M-EDTA, 0.05 M-Tris–HCl pH 8.0 was added and then mixed gently by inversion. The lysate was centrifuged at 30000g at 4 °C for 45 min. The fusion proteins were purified from the supernatant using affinity chromatography on Sepharose-bound IgG (Pharmacia). After washing, the fusion proteins were eluted with 0.1 M-glycine–HCl pH 2.4, and immediately neutralized with Tris base.

In order to establish whether the MV NP synthesized in *E. coli* retained the three known antigenic sites identified on the virus-induced protein, a recombinant plasmid was constructed which expressed a fusion protein representing all but the first 22 amino acids of the NP. This protein would have a theoretical Mr of 88K, i.e. 31K of Protein A plus 57K of NP. Examination by SDS–PAGE of the purified protein synthesized in *E. coli* showed that there was a range of polypeptides from 31K to 88K (data not shown). Western blot analysis using MAbs representing the three epitopes (MAbs 25, 105 and 120), showed that all three epitopic sites were present on the undegraded form (88K) of the fusion protein (Fig. 1). Only MAb 120 identified the majority of the degradation fragments, suggesting that the latter reacts with a site closer to the N terminus.

In order to determine the position of the antigenic sites of the MV NP, a series of deletions of the NP cDNA were made. Two strategies were used to construct the deletions. First, the NP gene was digested from its 3' end with exonuclease III. Second, internal fragments were removed by restriction enzymes (but retaining the reading frame), using either the two KpnI sites (nucleotide positions 410 and 554) or the two ApaI sites (nucleotide sites 248 and 1415). In some cases, a combination of the two methods was used. The constructions are summarized in Fig. 2.

Removal of the first seven amino acids of the C terminus of the NP (NP 23.476) leads to loss of reactivity with MAb 105, but not with the other two MAbs (Fig. 1). This region corresponds to that previously designated epitope III (Giraudon et al., 1988). Expression of an NP gene in which the *Apal* to *Apal* fragment (amino acids 68 to 456) had been removed [NP 23(68.456)525] gave a polypeptide of the predicted size which reacted with MAbs 25 and 105, but not with 120 (Fig. 1). Thus, MAb 25 recognizes a site between amino acid 457 and the C terminus. A more precise location of this site was obtained by examining the expression products of other constructs which had been partially digested from the 3' end of the NP gene. An NP protein deletion that terminated at amino acid 476 (NP 23.476) retained its reactivity with MAb 25 (Fig. 1). Thus the site recognized by MAb25 can be delimited by amino acids 457 and 476. This corresponds to epitope II (Giraudon et al., 1988).

In order to define the remaining antigenic site (MAb 120), we examined the proteins expressed from pRIT.NP constructs which had been more extensively digested with exonuclease III. The outer limits of the antigenic site were defined by NP 23.150 being positive...
Short communication

Fig. 1. Western blot analysis of MV NP expressed in E. coli. The NP cDNA or deletions of it (see Fig. 2) were cloned into the pRIT vector and a fusion protein was expressed. After purification, the NP proteins [lanes 1, 23.525; lanes 2, 23.518; lanes 3, 23.476; lanes 4, 23.150; lanes 5, 23.98; lanes 6, 23(67.457)525] were analysed with MAbs 120 (a), 25 (b) and 105 (c) (Giraudon & Wild, 1981; Giraudon et al., 1988). The MrS of marker proteins are shown on the left-hand side of the gels. For Western blot analysis (d), proteins were separated in 10% polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose paper [lanes 1, 23.525; lanes 2, 23(122.168)525]. The electroblots were incubated at room temperature with 10% MV-negative human serum, 10% bovine serum albumin in 0.2% Triton X-100 in order to block the Fc receptor of the Protein A moiety. MAbs directed against one of the three epitopes of the NP were added and incubated at room temperature for 90 min. The antigen-antibody complexes were revealed with an anti-mouse antibody complexed to peroxidase and dianimobenzidine tetrahydrochloride activated by hydrogen peroxide.
Fig. 2. Structure (hydrophathy) of MV NP and its deletions. The nomenclature of the clones represents their NP coding potential. MV NP mRNA was cloned by the method of Okayama & Berg (1982). A 1690 bp *BamHI* fragment was subcloned into pUC-18. The fragment contains the entire NP gene except for 119 bp at the 5' end and also contains 100 bp of vector DNA at the other extremity. This strategy makes use of the pUC polylinker in order to transfer the fragment into the *XmaI/PstI* site of the pRIT expression vector (Pharmacia) while keeping the open reading frame. A nested set of deletion mutants consisting of progressive removal of 3’ sequence of the NP gene was constructed by the use of exonuclease III and mung bean nuclease (Stratagene) according to the manufacturer's instructions. Briefly, pRIT. 2. NP was digested to completion with *XbaI* and *PstI*. The *PstI* cut provides a 3' single-stranded overhang which is not digested by the exonuclease; the *XbaI* cut provides a 5' single-stranded overhang which is the starting point for unidirectional removal of sequence from the *XbaI* site, which is situated 5 bp from the stop at the 3' end of the NP gene. Different incubation times were used with exonuclease III in order to obtain a nested set of 3' deletions and then mung bean nuclease was used to digest the ssDNA and create blunt ends. After recircularization of the DNA by ligation with T4 DNA ligase (Bethesda Research Laboratories), the constructs were transformed into N 1876 *E. coli*. Minipreparations were made from transformants using standard methods and analysed using restriction enzymes in order to determine the extent of the deletions. Large scale plasmid preparations of selected deletions were then prepared by standard methods (Maniatis *et al.*, 1982).

and NP 23.98 being negative with MAb 120. This situates the site between amino acids 98 and 150. The location of the site was further investigated by removing the *KpnI* to *KpnI* fragment (amino acids 122 to 168) from the (complete) pRIT NP construction. The protein expressed from this construct [NP 23(122.168)525] contained antigenic sites II and III, but lacked reactivity with MAb 120 (Fig. 1*d*). Thus, this antigenic site which we have designated site I lies between amino acids 122 and 150. The reactivity of the MAbs with the different constructs are summarized in Table 1.

We have located the regions on the MV NP antigen corresponding to the three antigenic domains previously identified (Giraudon *et al.*, 1988). The amino acid sequences are shown in Table 2. Epitope I as defined by MAb 120 reacts in all virus strains examined to date, whereas
Table 1. Summary of MV MAb reactivity with the proteins expressed in E. coli from different NP deletions

<table>
<thead>
<tr>
<th>NP</th>
<th>MAb 120</th>
<th>MAb 25</th>
<th>MAb 105</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.525</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23.518</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>23(67.457)525</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23.476</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>23.150</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>23.98</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>23(122.168)525</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Amino acid sequence of the antigenic sites mapped on the MV NP

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><strong>NMEDADQYFSHDDP</strong>ISSDQSRFGWFENK**</td>
</tr>
<tr>
<td>II</td>
<td><strong>SRASDARAAHLPT</strong>GTPLDID**</td>
</tr>
<tr>
<td>III</td>
<td><strong>NRNLD</strong></td>
</tr>
</tbody>
</table>

* The epitopes were defined by reactivity of MAbs 120 (epitope I), 25 (epitope II) and 105 (epitope III).
† The asterisks indicate amino acids identical to those in the canine distemper virus NP at the corresponding position.

the reactivity of the MAbs defining sites II (MAb 25) and III (MAb 105) varied with the virus isolate. Studies on influenza A virus NP have shown that this NP also possesses at least three non-overlapping antigenic sites, one being the same on all strains tested (van Wyke et al., 1980). This may suggest that the restraints on variation in the two viruses are similar.

The NP of the MV strain used for the construction of the expression plasmids in the present study (Halié) differs by 10 amino acids (R. Buckland, unpublished observations) from that published for the Edmonton strain (Rozenblatt et al., 1985; Cattaneo et al., 1988). None of the amino acid differences between the strains occurred in the proposed antigenic sites. In agreement with this, the three MAbs reacted equally well with both MV strains. The availability of sequence data for the NP gene from a number of paramyxoviruses has permitted comparison of the conserved elements. Although the conservation of amino acids varies greatly, both overall and within regions, analysis of the protein structure reveals that the hydropathy is well conserved (Sanchez et al., 1986). When viruses are more closely related, such as Sendai virus and parainfluenza 3 virus, the greatest conservation is in the hydrophobic regions, whereas the diversity occurs in two hydrophilic regions, one found at the C terminus and the other nearer the N terminus. These correspond to the same regions in which we have identified the three epitopes on the MV NP. Previous studies have shown an immunological cross-reaction between the NPs of MV and canine distemper virus (Hall et al., 1980; Örvell & Norby, 1980). Despite this cross-reactivity with polyclonal antiserum, the three MV MAbs used in our study did not cross-react with the Onderstepoort strain of canine distemper virus (Giraudon & Wild, 1981; P. Giraudon, unpublished results). Comparison of the amino acid sequences of MV epitope I with that of canine distemper virus (Rozenblatt et al., 1985) reveals that although it is in a region of high conservation only 38% of the amino acids within the predicted site are conserved. The other two epitopes are in a region of low conservation (8 to 15%), although four of the seven amino acids of site III are identical. Thus, the amino acid sequence data support our
Short communication

observations of a lack of cross-reactivity of the three MAbs used in the study with canine distemper virus.

The topographical arrangement of the NP within viral ribonucleoprotein particles of several paramyxoviruses has been studied by protein digestion. Trypsin cleavage of the NP in such particles of MV or Sendai virus gives an N-terminal 48K polypeptide which remains attached to the genome and a 12K C-terminal polypeptide which is liberated (Heggeness et al., 1981; Giraudon et al., 1988). Based on sequence and structural analyses of the Sendai virus NP, it has been proposed that the highly conserved hydrophobic regions within the 48K fragment are buried in the tertiary structure of the protein, possibly serving in non-covalent interaction with neighbouring NP molecules (Morgan et al., 1984; Jambou et al., 1986). The C-terminal fragment, which is rich in negatively charged amino acids, contains most of the phosphorylated amino acids (Hsu & Kingsbury, 1982) and protrudes from the virus particle. Antigenic analysis of the Sendai virus NP revealed two topographically distinct sites, both of which lie on the trypsin-digested bound NP (Deshpande & Portner, 1984). Our studies suggest that one of these sites corresponds to the MV site I, but that MV sites II and III are associated with the trypsin-sensitive C-terminal fragment (Giraudon et al., 1988).

Our approach to locating the antigenic sites on the MV NP is dependent upon MAbs that recognize the antigen in a Western blot analysis. Attempts to extend the technique to the MV haemagglutinin and fusion proteins were unsuccessful, as our MAbs did not identify the denatured antigen. This may be because the epitopes on these antigens are conformation-dependent, whereas those on the NP are linear. In this respect, it has been shown that a number of the cysteine residues are highly conserved in the haemagglutinin and fusion proteins of paramyxoviruses (Alkhatib & Briedis, 1986; Buckland et al., 1987). These would impose configurational restraints that would be lost during Western blotting. In contrast, the MV NP contains a single cysteine residue. In this case, conformational epitopes would depend more on protein–protein interaction than on disulphide bridges.

Our studies have localized three of the epitope regions on the MV NP. We are now in the process of identifying the corresponding sites on field strains which show antigenic diversity. Using these data, it will be possible to use synthetic peptides representing the different epitopes to examine the role of MV variants in various pathological states.

We would like to thank Lucia Monaco (Milan) for advice on the expression of the pRIT constructions and the consequent preparations of antigen, and M. F. Jacquier for the photography.

REFERENCES


(Received 9 June 1988)