Detection and characterization of proteins encoded by the second ORF of the M2 gene of pneumoviruses

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The nucleotide sequence of the M2 gene of pneumonia virus of mice (PVM) was determined. The sequence showed that the gene encoded a protein of 176 amino acids with a predicted molecular mass of 20165 Da from a major ORF, which is smaller than the equivalent proteins encoded by human, bovine and ovine respiratory syncytial (RS) viruses. The PVM M2 protein is conserved, having 41% similarity to the equivalent human RS virus protein. In common with the M2 genes of the RS viruses and avian pneumovirus (APV), the PVM mRNA also contained a second ORF (ORF2) that partially overlaps the first ORF and which is capable of encoding a 98 residue polypeptide. No significant sequence identity could be detected between the putative M2 ORF2 proteins of PVM, APV and the RS viruses. The expression of the M2 ORF2 proteins of the pneumoviruses was investigated by using monospecific antisera raised against GST fusion proteins. Western blot analysis demonstrated the presence of polypeptides encoded by M2 ORF2 of PVM and RS virus corresponding with those predicted by in vitro translation studies, but this was not the case for APV. The PVM polypeptide was present as three distinct products in vivo. The PVM and RS virus polypeptides were also detected in cells by immunofluorescence, which showed that both were present in the cytoplasm with a degree of localization in inclusion bodies. No APV M2 ORF2 protein could be detected in vivo. The RS virus M2 ORF2 polypeptide was shown to accumulate during infection and the potential implications of this are discussed.

Introduction

Pneumoviruses represent a distinct genus of the family Paramyxoviridae and are responsible for infections of the respiratory tract in their respective hosts, with human respiratory syncytial (RS) virus being the major cause of paediatric respiratory tract disease worldwide. The other members of the genus Pneumovirus are pneumonia virus of mice (PVM) and avian pneumovirus (APV). Several features distinguish RS virus from most other members of the family Paramyxoviridae, and it has been designated as the type member of the genus Pneumovirus of the sub-family Pneumovirinae. A major feature is the difference in genome organization between pneumoviruses and other paramyxoviruses. In particular, mammalian pneumovirus genomes contain ten genes, compared with six or seven for the paramyxovirus, rubulavirus and morbillivirus genomes, and sequences of pneumovirus proteins display virtually no amino acid similarity to those of other viruses outside the genus, with the exception of the L and F proteins, which both show a low but significant level of similarity (Stec et al., 1991; Yu et al., 1991; Chambers et al., 1992; Randhawa et al., 1996). APV contains a total of eight genes and the gene order is different from those of the mammalian pneumoviruses (Yu et al., 1992; Ling et al., 1992; Randhawa et al., 1997). A second distinguishing feature of pneumoviruses is the apparent limited utilization by RS virus of alternative ORFs within mRNA. In contrast, several paramyxoviruses, such as Sendai virus and measles virus, access alternative ORFs within the mRNA encoding the phosphoprotein to direct the synthesis of a novel protein(s) (Bellini et al., 1985; Galinski et al., 1986; Kondo et al., 1990). Analysis of PVM, a pneumovirus closely related to RS virus, has shown that this characteristic of pneumoviruses may not be absolute, as the P gene contains, and expresses, a second ORF and several additional proteins are synthesized by using internal AUG initiation codons within the P protein ORF (Barr et al., 1994).

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Sequence analysis of other pneumovirus genes has shown that some mRNAs contain a second, small, alternative ORF. Of particular note is the presence of a second ORF (ORF2) in the mRNA encoding the M2 proteins of all RS viruses and APV (Collins & Wertz, 1985; Elango et al., 1985; Baybutt & Pringle, 1987; Collins et al., 1990; Ling et al., 1992; Zamora & Samal, 1992; Alansari & Potgieter, 1994). Each of these internal ORFs partially overlaps the upstream major ORF. These second ORFs show conservation in location but not in the predicted sequence of the putative polypeptide. This striking degree of conservation suggests that the alternative ORFs may be functional. Studies involving the rescue of infectious RS virus from a full-length cDNA clone have suggested that the product of the second ORF of the M2 gene plays a role in transcription and replication of the virus genome (Chambers et al., 1995, 1996; Hardy & Wertz, 1998). To date the polypeptide products of these second ORFs have not been detected directly. We report here the sequence of the M2 gene of PVM and the identification of polypeptides encoded by ORF2 of the pneumovirus M2 genes, together with their localization within the infected cell.

Methods

- **Cells and viruses.** Human RS virus strains A2 and S2, PVM strain 15 and APV strain CVL/14 were propagated in BSC-1 cells.

- **Nucleic acid manipulations.** mRNA was extracted from infected BSC-1 cells as described previously (Ling et al., 1992). The production and preliminary characterization of cDNA clones has been described previously (Chambers et al., 1990). Clones representing the M2 gene were isolated and the complete nucleotide sequence determined. For PCR amplification, cDNA was prepared by using oligo(dT) as a primer and aliquots were used to amplify the regions of the M2 gene containing ORF2. The entire RS virus M2 gene was amplified by using primers RS22K1A (5′ GGGAATTCGCCAATATGTCACG-3′) and RS22K7 (5′ GGTTCTACGGATCATTTTGGTCACACAC-3′) and ORF2 alone was amplified by using primers RS22K6 (5′ GGGAATTCGGTACATAATGACC-3′) and RS22K7. The entire PVM M2 gene was amplified by using primers PVM22K1A (5′ GGGAATTCAGGATGAGTGTGAGACAA-3′) and PVM22K6 (5′ CGAGGCTACCCGATACGTCCACA-3′) and ORF2 alone was amplified with primers PVM22K5 (5′ TACGGACCTCAGGATATGAC-3′) and PVM22K6. The entire APV M2 gene was amplified with primers APV22K1A (5′ GGGAATTCGCCAATATGTCACG-3′) and APV22K4 (5′ GACCTGCGAATTACTAATTATATGCTGG-3′) and ORF2 alone was amplified with primers APV22K3 (5′ CCCGGGTACAAAATGCTGG-3′) and APV22K4. The numbers refer to the 3′ nucleotide in the gene to which the oligonucleotides anneal and restriction enzyme cleavage sites included for cloning purposes were underlined. PCR products were purified and inserted into appropriate plasmid vectors by using the restriction endonucleases for which recognition sites had been included in the PCR primer oligonucleotides and the nucleotide sequence of each insert was confirmed. The DNA fragments representing ORF2 of the M2 genes were inserted into the multiple cloning site of the expression vector pGEXX-1 (Pharmacia) between the EcoRI and SalI recognition sites. In this way, the putative M2 ORF2 protein was expressed as a carboxy-terminal extension of GST.

- **Generation of polyclonal antisera and indirect immunofluorescence.** The purified GST fusion proteins were used to raise polyclonal antisera after injection into rats (for PVM and APV) and rabbits (for RS virus). The sera were shown to detect the bacterially expressed proteins by Western blot analysis (not shown). For immunofluorescence, BSC-1 cells grown on coverslips were infected with RSV at an m.o.i. of 1 p.f.u. per cell or with PVM and APV at an m.o.i. of 0.1 p.f.u. per cell and incubated at 37 °C until a cytopathic effect was visible. The cells were washed three times with PBS and fixed with a 1:1 (v/v) mixture of acetone and methanol (precooled at –20 °C) for 20 min at room temperature. After three washes with PBS, the cells were incubated with the primary antibody for 1 h at 37 °C. The antibody was removed and the cells were washed three times with PBS, after which they were incubated with the appropriate biotinylated anti-species antibody, diluted 1:400 in PBS, for 1 h at 37 °C. The cells were washed as before and incubated with streptavidin–FITC conjugate for 15 min at 37 °C in the dark. The cells were washed three times with PBS and then fixed in 80% glycerol in PBS and fluorescence was visualized.

- **Preparation of antigen for Western blot analysis.** Twenty-four hours after infection, BSC-1 cells were washed twice with PBS, scraped into PBS, pelleted by centrifugation and lysed in lysis buffer (0.6% NP40, 150 mM NaCl, 1.5 mM MgCl2, 10 mM Tris–HCl). The proteins were separated by PAGE and transferred to filters for Western blot analysis.

- **In vitro transcription and translation.** The M2 genes were transferred into pBluescribe plasmids such that they were under the control of the bacteriophage T7 promoter. RNA was used to direct the synthesis of proteins in a rabbit reticulocyte translation system (Amersham) as described previously (Chambers et al., 1990). The synthesized polypeptides were labelled by the addition of [35S]methionine in the translation reaction. The presence of globin in the reticulocyte lysates affected the apparent molecular mass of the M2 ORF2 proteins. In order to avoid this, the products were immunoprecipitated as described by Ling & Pringle (1989a, b) and were subsequently analysed by PAGE followed by detection by autoradiography.

Results and Discussion

The complete nucleotide sequence of the M2 gene of PVM strain 15 is shown in Fig. 1. The gene contains a major ORF, beginning at the 5′-proximal AUG initiation codon, capable of encoding a protein of 176 amino acids with a predicted molecular mass of 20165 Da. This is somewhat smaller than the equivalent proteins encoded by human RS viruses (194 residues for subgroup A and 195 residues for subgroup B) (Elango et al., 1985; Collins & Wertz, 1985; Baybutt & Pringle, 1987; Collins et al., 1990) and bovine and ovine RS viruses (186 residues) (Zamora & Samal, 1992; Alansari & Potgieter, 1994). The PVM M2 protein has 41% similarity to the human RS virus M2 protein and comparison of all pneumovirus M2 proteins showed that most of the conservation was located in the amino-terminal half (not shown), as noted for the RS virus M2 proteins (Collins et al., 1990; Zamora & Samal, 1992). As is the case for the other M2 proteins, the PVM M2 protein contains a high proportion of cysteine residues at the amino terminus. Such a concentration of cysteines is frequently found in zinc-binding proteins. Further analysis will be necessary to
Pneumovirus M2 ORF2 proteins

Fig. 1. Nucleotide sequence of the PVM M2 mRNA. The sequences of the deduced polypeptides are also shown.

establish whether this is relevant to the function of the M2 proteins. A striking feature of the sequences of the RS virus M2 genes was the conservation of a second ORF that partially overlaps the first ORF, and which is capable of encoding a polypeptide ranging in size from 90 to 95 amino acids depending on the virus (Elango et al., 1985; Collins & Wertz, 1985; Baybutt & Pringle, 1987; Collins et al., 1990; Zamora & Samal, 1992; Alansari & Potgieter, 1994). A second ORF capable of encoding a polypeptide of 73 amino acids was also identified in the M2 gene of APV (Ling et al., 1992). A second ORF (ORF2) was also present in the M2 gene of PVM (Fig. 1), and this could direct the synthesis of a 98 residue polypeptide. As with the other pneumoviruses, ORF2 partially overlapped ORF1. While the ORF2 proteins of the various RS viruses were similar, no significant sequence identity could be detected between the putative M2 ORF2 proteins of PVM, RS virus and APV.

The sera from animals immunized with a GST fusion protein containing the putative product of ORF2 of the RS virus M2 gene detected a polypeptide with an apparent molecular mass of approximately 9.3 kDa in the RSV-infected cells that was absent in mock-infected cells (Fig. 2). The observed mobility of this protein on SDS–PAGE corresponds, though not perfectly, with the molecular mass predicted from the nucleotide sequence (10 675 Da) and with the approximately 8.5 kDa polypeptide seen when the M2 ORF2 was used to direct the translation of a polypeptide product in vitro (Fig. 3). This may indicate that the polypeptide produced in vivo is subject to post-translational modification. To confirm that expression of this protein is not restricted to strain A2, the RS virus M2 ORF2-specific antiserum was used in a Western blot to confirm expression of the analogous protein from the RS virus strain S2. The predicted proteins encoded by ORF2 of the M2 gene in both strains of RSV are very similar, with 88% amino acid identity between the two strains (Tolley et al., 1996). These two proteins have 10 amino acid differences and the protein in strain A2 also has two more amino acids at the amino terminus. Expression of an ORF2 polypeptide was also seen in RS virus S2-infected cells (Fig. 2).

Using sera raised against the GST–PVM M2 ORF2 fusion protein, Western blot analysis detected three proteins with approximate molecular masses of 13.5, 16 and 17 kDa (Fig. 2) that were absent in the mock-infected cells. The smallest polypeptide, which was consistently detected as a fainter band than the others, has a mobility corresponding closely with the observed molecular mass (14 kDa) of the PVM M2 ORF2 protein translated in vitro with a rabbit reticulocyte lysate (Fig. 3), though this is larger than the predicted molecular mass of 11 503 Da. The origin of the two larger polypeptides is not clear and they may be post-translationally modified versions of the smaller protein. It is interesting to note that the first initiation codon of ORF2 is in a poorer context for translation than the second, which overlaps the stop codon of the first ORF.

Sera raised against a fusion protein containing sequences encoded by the APV M2 ORF2 failed to detect a polypeptide in infected cells, though it precipitated a polypeptide of approximately 8.6 kDa from an in vitro translation (Fig. 3). It is possible that this is due either to lack of expression of this ORF in vivo or to the protein product being expressed at levels below those that are detectable using this assay.

The monospecific antisera raised against the three fusion proteins were also used to detect the possible expression of the products of ORF2 in virus-infected cells by immunofluorescence. As seen with the Western blot analysis, the APV ORF2-specific antibody failed to provide any evidence for
Fig. 2. Detection of the RS virus and PVM M2 ORF2 proteins. Western blots probed with antisera raised against GST fusion proteins with the putative RS virus and PVM M2 ORF2 proteins are shown. The pre-immune serum reaction with infected and mock-infected extracts are also shown. Protein extracts were prepared from mock-infected cells and cells infected with RS virus strains A2 and S2 and with PVM, as indicated. The M2 ORF2 proteins are identified by asterisks and the sizes and positions of standard molecular mass markers are shown (kDa).

Fig. 3. In vitro translation products of the pneumovirus M2 genes. Autoradiograph of a polyacrylamide gel showing \([^{35}S]m\)ethionine-labelled in vitro translation products of the M2 genes of RSV (lane 1), APV (2) and PVM (3) containing ORF1 and ORF2 after immunoprecipitation with antisera raised against the relevant ORF2–GST fusion proteins. The result of translation and precipitation of a minus-RNA control is shown (lane 4). The positions of size markers (in kDa) are shown.

expression of this protein in vivo. As before, this may be due to either the absence or very low levels of expression of the APV M2 ORF2 protein. In contrast, the antisera directed against the ORF2 products of the M2 genes of PVM and RS virus gave positive fluorescence signals in the infected cells (Fig. 4). No fluorescence was seen with mock-infected cells or with infected cells treated with pre-immune serum. In RS virus-infected cells, fluorescence was visible in the cytoplasm, with some evidence of localized cytoplasmic inclusions. There was frequently a higher degree of fluorescence in the vicinity of the nucleus. With the PVM-specific antiserum, as with the anti-RS virus serum, no fluorescence was seen with mock-infected cells. In PVM-infected cells, immunofluorescence was mostly distributed diffusely in the cytoplasm. However, some particulate forms of fluorescence could be seen, suggesting the increased localization of the PVM M2 ORF2 protein in specific regions.

Fig. 4. Cellular localization of the M2 ORF2 proteins of PVM and RS virus by immunofluorescence. (A) PVM-infected cells. (B) RS virus-infected cells.
of the cytoplasm. The small numbers of cytoplasmic inclusions containing the PVM and RSV proteins may indicate the presence of aggregates of these proteins or the presence of an interaction(s) with other virus or infected-cell proteins. Most of the inclusion bodies in RSV-infected cells are thought to be aggregates of virus nucleocapsids (Garcia et al., 1993).

The data presented here show that the mammalian pneumoviruses direct the synthesis of polypeptides from ORF2 present in the M2 gene. Since this region of the genome has been associated with the inhibition of virus RNA synthesis in the rescue of synthetic RS virus replicons, it is likely that the PVM polypeptide also carries out the same function, although this remains to be established. The inability to detect an equivalent polypeptide in APV-infected cells may indicate a significant difference between the mammalian pneumoviruses and the newly named genus Metapneumovirus (Pringle, 1998). APV has been shown to have significant differences in the number of genes and genome organization when compared with the other pneumoviruses (Ling et al., 1992; Yu et al., 1992; Randhawa et al., 1997), and this may have a bearing on the absence of the M2 ORF2 protein. However, it is possible that the levels of expression from this ORF are very low in APV-infected cells and are below the level of detection. The antiserum used was shown to be able to detect bacterially expressed protein with approximately the same degree of sensitivity as the sera raised against the PVM and RS virus proteins (not shown). The data presented here, and other data from genetic studies and analysis of the expression of the PVM P gene, clearly show that pneumoviruses, as is the case for other members of the family Paramyxoviridae, can utilize second ORFs to direct the synthesis of additional polypeptides and in this way can increase the coding capacity of their genomes.

The kinetics of synthesis of the M2 ORF2 protein in RSV-infected cells was analysed by Western blot analysis of samples prepared at 12, 18, 24 and 48 h post-infection (p.i.) (Fig. 5). The RSV M2 ORF2 protein could not be detected at 12 or 18 h p.i., was initially detected at 24 h p.i. and continued to accumulate throughout the infectious cycle to 48 h. This pattern of accumulation as the infection proceeds has been described for the RSV structural polypeptides (Lambert et al., 1988) and the non-structural NS1 protein but contrasts with the NS2 protein, which has been shown to turn over rapidly during infection (Evans et al., 1996). The low level of expression at early times in infection may reflect the relative use of the M2 gene ORF2, not only because of the position of the M2 gene on the virus genome relative to the promoter at the 3′ end, but also because the location of this ORF in the M2 gene mRNA transcript may mean that it is only rarely accessed by ribosomes. The increasing amount of this protein over a long time during infection suggests that it is stably accumulated in the infected cells and may indicate that this protein is needed in the late stages of the virus life-cycle. Together with the observation that the M2 ORF2 protein inhibits transcription from the RS virus genome in a concentration-dependent manner (Collins et al., 1995, 1996), these data suggest that the protein may possibly act as the trigger, when an appropriate intracellular concentration is achieved, to precipitate the virus from a transcriptional mode into assembly of progeny nucleocapsids suitable for maturation into infectious virus particles, and is consistent with the observation that transfection of a plasmid expressing the M2 ORF2 protein enhanced packaging of a synthetic minigenome (Teng & Collins, 1998). Elucidation of the role of these proteins in the life-cycle of the pneumoviruses may provide further insights into the mechanisms by which these viruses control their gene expression.

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References


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