Identification of residues critical for the human coronavirus 229E receptor function of human aminopeptidase N

Andreas F. Kolb, Annette Hegyi and Stuart G. Siddell

Institute of Virology and Immunology, University of Würzburg, Versbacherstrasse 7, 97078 Würzburg, Germany

Aminopeptidase N (APN) is the major cell surface receptor for group 1 coronaviruses. In this study, we have isolated and characterized a feline APN cDNA and shown that the transfection of human embryonic kidney cells with this cDNA renders them susceptible to infection with the feline coronavirus feline infectious peritonitis virus, the human coronavirus (HCV) 229E and the porcine coronavirus porcine transmissible gastroenteritis virus. By using chimeric APN genes, assembled from porcine and feline sequences, we have shown that, analogously to the human APN protein, a region within the amino-terminal part of the feline APN protein (encompassing amino acids 132–295) is essential for its HCV 229E receptor function. Furthermore, by comparing the relevant feline, human and porcine APN sequences, we were able to identify a hyper-variable stretch of eight amino acids that are more closely related in the feline and human APN proteins than in the porcine APN molecule. Using PCR-directed mutagenesis, we converted this stretch of amino acids within the porcine APN molecule to the corresponding residues of the human APN molecule. These changes were sufficient to convert porcine APN into a functional receptor for HCV 229E and thus define a small number of residues that are critically important for the HCV 229E receptor function of human APN.

Introduction

Coronaviruses are enveloped, positive-strand RNA viruses that are primarily associated with respiratory and gastrointestinal disorders in both animals and man. The family Coronaviridae is divided into two genera, the coronaviruses and the toroviruses, and the coronavirus genus can be further divided into three groups, 1, 2 and 3 (Siddell, 1995). Group 1 comprises five viruses; namely, human coronaviruses related to strain 229E (HCV 229E), porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhoea virus (PEDV), canine coronavirus (CCV) and feline infectious peritonitis virus (FIPV). These viruses constitute a distinct genetic cluster (Siddell, 1995) and four of them, HCV 229E, TGEV, PEDV and FIPV, have been shown to use aminopeptidase N (APN) as a cell surface receptor (Delmas et al., 1992; Yeager et al., 1992; Tresnan et al., 1996).

APN is a type II glycoprotein that belongs to the family of membrane-bound metalloproteases (Olsen et al., 1988). APN is expressed in a variety of tissues, including cells of the granulocyte and monocyte lineage, fibroblast, synaptic membranes of the CNS and epithelial cells from the renal proximal tubules, intestinal brush border and the respiratory tract (Tresnan et al., 1996). The tissue distribution of APN is clearly a factor in the pathogenesis of coronavirus infections but it is certainly not the only determinant. HCV 229E, TGEV and FIPV infections lead to very distinct diseases, affecting different host tissues (de Groot & Horzinek, 1995; Myint, 1995; Garwes, 1995). Also, the idea that, for any one coronavirus, infection is restricted to a single host, and this restriction is largely determined by the specificity of the virus–receptor interaction, can no longer be sustained. It is true that human APN (hAPN, also known as CD13) and porcine APN (pAPN) only mediate the infection by HCV 229E and TGEV, respectively but, recently, it has been shown that feline APN (fAPN) can serve as a receptor not only for FIPV but also for TGEV, HCV 229E and CCV (Tresnan et al., 1996). Consequently, cats can be infected with all of these viruses, although only infection with the feline coronavirus FIPV (or the feline enteric coronavirus variant, FECV) gives rise to overt disease (Barlough et al., 1984, 1985).

We are interested in defining the interaction between hAPN and the surface glycoprotein of HCV 229E, as this is an obvious target for the development of drugs that can be used to inhibit the infection process. In previous studies, we have used chimerical hAPN/pAPN molecules to define a region (amino acids 260–353) that is specifically involved in the HCV
229E receptor function of hAPN (Kolb et al., 1996). In the course of these studies, we also noticed that feline kidney cells could be infected with HCV 229E. We assumed, and it has subsequently been shown by Tresnaj et al. (1996), that feline APN (fAPN) can also serve as a receptor for HCV 229E and this assumption led us to make two predictions. First, we would predict that, analogously to hAPN, the fAPN region which mediates HCV 229E reception is located within the amino-terminal part of the protein. This is in contrast to, for example, the region of the pAPN molecule which is essential for TGEV infection, i.e. the carboxyl-located amino acids 716–813 (Delmas et al., 1994). Second, we would predict that a comparison of the relevant hAPN and fAPN sequences will help us to define more precisely the hAPN amino acids that are critical for HCV 229E reception. In this paper, we show that both of these predictions are correct and this information has been used to identify, by mutational analysis, a stretch of eight amino acids within the hAPN molecule that is critically involved in its function as a receptor for HCV 229E.

Methods

**Viruses and cells.** MRC-5 cells (ECACC 84101801) were grown at 37 °C in minimal essential medium (Life Technologies) supplemented with 10% FBS, non-essential amino acids, glutamine and antibiotics. Crandell feline kidney cells (CRFK; ECACC 86093002), swine testis cells (ST, ECACC 92040221), Felis catus whole foetus cells (Fcowf, Pedersen et al., 1981), COS-7 cells (ECACC 87021302) and 293 human embryonic kidney cells (293 HEK, ECACC 85120602) were grown at 37 °C in Dulbecco’s modified MEM (Sigma) supplemented with 10% FBS, non-essential amino acids, glutamine and antibiotics. The HCV 229E isolate used in these studies has been described previously (Raabe et al., 1990) and was propagated in monolayers of MRC-5 cells at 33 °C. The Purdue strain 46 of TGEV was propagated in ST cells. FIPV strain 79-1146 was used and was propagated in monolayers of MRC-5 cells at 33 °C and was propagated in Fcwf cells. FIPV-infected cells were metabolically labelled 6–9 h post-infection with [35S]methionine/ml (Amersham).

**Protein analysis.** Cytoplasmic extracts of transfected/infected cells were prepared as described (Grosse & Siddell, 1994). The extracts isolated from HCV 229E- or TGEV-infected cells were separated on a 15% SDS–polyacrylamide gel, transferred to nitrocellulose and analysed by immune-staining with the HCV 229E nucleocapsid protein-specific antibody NG12 (Ziebuhr, 1995) or the TGEV nucleocapsid protein-specific antibody 3C/E4 (Sanchez et al., 1990). The immune reactions were developed with the ECL system (Amersham). Immune precipitations using the cytoplasmic extracts prepared from FIPV-infected cells were performed essentially as described (Grosse & Siddell, 1994). Briefly, extracts from 2 × 10^5 cells were mixed with 2 μl of an FIPV-specific antisemur (de Groot et al., 1987) in a total volume of 150 μl in NTPebuffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.2% NP40, 1 mM EDTA, 1 mg/ml BSA and incubated at 4 °C for 2 h. Then, 10 mg of Protein A–Sepharose was added and, after a further incubation of 1 h at 4 °C, the bound immune-complexes were collected by centrifugation. The immune-complexes were washed three times in NTPebuffer and eluted from the Protein A–Sepharose by incubation for 15 min at 56 °C in a buffer containing 60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.002% bromophenol blue and 25 mM DTT. After centrifugation, the eluted proteins were heated to 100 °C for 3 min and separated on a 15% SDS–polyacrylamide gel.

**Recombinant DNA.** A feline APN cDNA was isolated from the mRNA of CRFK cells by RT–PCR with primers corresponding to conserved regions of mammalian APN genes. Briefly, total RNA was isolated from CRFK cells as described previously (Kolb et al., 1994) and polyadenylated RNA was selected by poly(U)–Sepharose chromatography. Subsequently, cDNA was generated by reverse transcription with MLV-RT (Life Technologies) and oligo(dT) primer (PharMacia) for 1 h at 42 °C. The CDNA was then used as template for a PCR reaction with the primer pair 5′ CTCCCTGAGGCTACATGAAAAGGCGTTCTATATT 3′ (primer 1) and 5′ AAAGCCCTGTGAGCGCAATGTCATCTCCAAA 3′ (primer 23). Amplification was done with Pfu polymerase (Stratagene) at a primer concentration of 0.5 μM using 36 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C. The resulting PCR product of 1245 bp was cloned into the vector pBlueScript to generate pB-amino-APN and three independent clones were sequenced. From the DNA sequence, a feline APN-specific primer 24, 5′ TATGACAGGCTTCCCTGCCG 3′ was derived and used for a second PCR reaction together with primer 9, 5′ GGATCGATACATTGCTGTATTTCCTGTAACCA 3′. This PCR was done as described above using the same cDNA template and 38 cycles of 30 s at 94 °C, 30 s at 60 °C and 4 min at 72 °C. The resulting 1815 bp PCR product was cloned into the vector pBlueScript to generate the plasmid pB-carboxy-fAPN and three independent clones were sequenced.

To construct a full-length IAPN cDNA, a 1.2 kb Xhol–EcoRI fragment was excised from p8-amino-IAPN and cloned into the expression vector pBK-CMV(-) lac which had been digested with SauI and EcoRI. pBK-CMV(-) lac was derived from the vector pBK-CMV (Stratagene) by deletion of a 174 bp Nhel–SacI fragment encompassing the prokaryotic β-galactosidase promoter sequence. The plasmid pBK-CMV-amino-IAPN was subsequently digested with EcoRI and Xbal and a 1.8 kb EcoRI–Xbal fragment derived from pB-carboxy-fAPN was inserted. The construction of a complete fAPN cDNA was confirmed by sequencing and the resulting plasmid pBK-IAPN was used for transfection studies.

PCR reactions with 2 μg of CRFK and Fcwf genomic DNA and primers 27 (5′ GCCCAGTGCATCAACCA 3′; corresponding to codons 304–308 in the IAPN gene) and 23 were done at a primer concentration of 0.5 μM using 36 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C. Isolation of genomic DNA was done as described (Kolb & Siddell, 1996). To generate the expression vector AP34, a 494 bp Dnal–Bsal fragment of the IAPN cDNA was inserted into plasmid pUC-pAPN[B/S] that had been digested with the same enzymes. pUC-pAPN[B/S] contains a 1.5 kb fragment of the porcine APN cDNA encoding amino acids 1–500, cloned into pUC19. After insertion of the IAPN fragment, the modified pUC-pAPN[B/S] was digested with BamHI and SphI and the 1.5 kb fragment was re-inserted into plasmid AP1 (Delmas et al., 1994) that had been digested with the same enzymes. The resulting plasmid,
AP34, contains a complete pAPN open reading frame in which the pAPN amino acids 132–295 are replaced by amino acids 135–297 of fAPN.

To generate plasmid AP35, a 907 bp PCR product was amplified from pAPN cDNA by using primers 35, 5’ GAGTTCGACTACGTGGAGAAACAGGCTCAATGGCCGTCCTGATCCGGATCTGG 3’, and 25. The PCR product was digested with AccI and BsiBI. The resulting 259 bp fragment was cloned into pUC-pAPN/B/S previously digested with the same enzymes. The modified pUC-pAPN/B/S was digested with BamHI and SphI and the 1–5 kb fragment was re-inserted into plasmid AP1 that had been digested with the same enzymes. The resulting expression vector AP35 contains a complete pAPN open reading frame in which the pAPN amino acids 283–290 (QSVNETAQ) are replaced by the hAPN amino acids 288–295 (DYVEKQAS).

Results

Cloning and sequence analysis of CRFK fAPN cDNA

As a prerequisite for the construction of chimerical APN molecules containing fAPN sequences, we isolated a feline APN cDNA from CRFK cells by RT–PCR. The amino acid sequence deduced from the cDNA (GenBank accession number U96104) predicts a CRFK fAPN protein of 966 amino acids with sequence motifs characteristic of mammalian aminopeptidase N. These include an amino-terminal cytoplasmic tail (amino acids 1–9), an amino-proximal transmembrane domain (amino acids 10–32) and an extended ectodomain (amino acids 33–966). The metalloproteinase catalytic centre, with the highly conserved HELAH amino acid motif, is located between amino acids 386–390. Alignment of the predicted CRFK fAPN sequence with other APN proteins shows that the feline protein is most closely related to human and porcine APN, although the percentage similarity with all known mammalian APN proteins is in the range 75–78% (data not shown).

Tresnan et al. (1996) have reported the sequence of an fAPN cDNA isolated from Fcwf cells. Comparison of the predicted CRFK fAPN protein and the predicted Fcwf fAPN protein shows, as expected, a close relationship, with 98% amino acid similarity (data not shown). The two proteins do, however, differ in length, 966 and 967 amino acids, respectively, and there are 16 amino acid exchanges. This close relationship is also reflected by a PCR analysis using genomic DNAs from CRFK and Fcwf cells (Fig. 1). In both cases, a PCR product of 632 bp could be obtained using a primer specific for the fAPN cDNA sequence (primer 27) combined with a primer that corresponds to a sequence highly conserved between mammalian APN cDNAs (primer 23).

Using the same primers, a PCR product of 323 bp could be also detected when pBK-CMV-fAPN DNA was used as template, indicating that the 632 bp PCR product derived from genomic DNA contains 309 bp of intron sequence. Sequence analysis of the PCR product derived from genomic DNA (GenBank accession number U96105) revealed that two introns are contained within the amplified region. As expected, no PCR product was obtained with genomic DNA from MRC-5 cells (human), ST cells (porcine) and COS-7–hAPN cells (African green monkey cells stably transfected with the plasmid pBK-CMV-hAPN; Kolb et al., 1996), confirming that the APN cDNA we have isolated is, indeed, of feline origin.

Receptor function of fAPN

The CRFK fAPN cDNA was introduced into a eukaryotic expression vector and transfected into 293 HEK cells. As controls, 239 HEK cells were also transfected with vectors that express hAPN and pAPN. The cells were then challenged with FIPV, labelled metabolically with $\[^{35}\text{S}\]_{\text{methionine}}$ and the FIPV structural proteins were immune-precipitated using a specific antiserum. The results, shown in Fig. 2(a), demonstrate that transfection of the vector pBK-CMV-fAPN rendered 293 HEK cells susceptible to FIPV infection and that the infection progressed, at least to the stage of viral protein synthesis. In accordance with previous results (Delmas et al., 1993) expression vectors directing the synthesis of human or porcine APN did not render 293 cells susceptible to FIPV infection. Identical results were also obtained with pools of stably transfected 293 cell clones (data not shown).
Fig. 2. (a) Immune-precipitation of FIPV structural proteins. 293 HEK cells were transiently transfected with the expression vectors pBK-CMV-fAPN, pBK-CMV-hAPN or AP1. The cells were challenged with FIPV at an m.o.i. of 5 and labelled metabolically with $^{35}$Smethionine. Cytoplasmic extracts of the transfected and infected cells were immune-precipitated with an FIPV-specific antiserum. The precipitated proteins were separated on a 15% SDS–polyacrylamide gel and visualized by autoradiography. The molecular masses of the $^{14}$C-labelled marker proteins (Amersham) and the positions of the FIPV-specific structural proteins (surface glycoprotein, S; nucleocapsid protein, N; membrane protein, M) are indicated. (b) Western blot analysis of HCV 229E-infected cells. 293 HEK cells were transiently transfected with the expression vectors pBK-CMV-fAPN, pBK-CMV-hAPN and pBK-CMV. The transfected cells and Fcwf cells were challenged with HCV 229E at an m.o.i. of 5. Cytoplasmic extracts of the transfected and infected cells were separated on a 15% SDS–polyacrylamide gel and transferred to nitrocellulose. The HCV 229E nucleocapsid protein was detected by immune-staining using the monoclonal antibody NG12. The molecular masses of the marker proteins (Pharmacia) and the position of the HCV 229E nucleocapsid protein (N) are indicated. (c) Western blot analysis of TGEV-infected cells. 293 HEK cells were transiently transfected with the expression vectors pBK-CMV-fAPN, AP1 and pBK-CMV. The transfected cells and Fcwf cells were challenged with TGEV at an m.o.i. of 5. Cytoplasmic extracts of the transfected and infected cells were separated on a 15% SDS–polyacrylamide gel and transferred to nitrocellulose. The TGEV nucleocapsid protein was detected by immune-staining using the monoclonal antibody 3C/E4. The molecular masses of the marker proteins (Pharmacia) and the position of the TGEV nucleocapsid protein (N) are indicated.
In a second series of experiments, shown in Fig. 2(b,c), we were able to confirm that fAPN can also function as a receptor for both HCV 229E and TGEV. The experiments were done essentially as described above, except that monoclonal antibodies were used to detect the accumulation of either HCV 229E or TGEV nucleocapsid proteins in the infected cells. We have previously shown that this assay is able to discriminate de novo protein synthesis (Kolb et al., 1996) and cells transfected with the control plasmid, pBK-CMV, were clearly negative. These data show that the fAPN cDNA isolated from CRFK cells encodes a receptor for FIPV, TGEV and HCV229E and is functionally identical to the fAPN cDNA isolated from Fcwf cells (Tresnan et al., 1996).

HCV 229E is usually propagated on cell lines with a limited life-span (e.g. MRC5 or WI38 cells) as permanently growing cell lines, even when they are permissive to HCV 229E infection (like HeLa cells or L929 cells transfected with an hAPN expression plasmid), fail to produce detectable titres of infectious virus (data not shown). Similarly, CRFK cells do not support the multiplication of HCV 229E. TGEV and FIPV, however, can be propagated on CRFK cells (data not shown), suggesting that maturation of HCV 229E requires an additional factor which is not essential for the production of infectious TGEV or FIPV virions.

**Receptor function of chimerical fAPN/pAPN proteins**

Amino acids 260–353 of the hAPN molecule are essential in determining the HCV 229E receptor function of the protein (Kolb et al., 1996). We have shown above that fAPN can serve as a receptor for HCV 229E and it seemed likely that the homologous region of the fAPN protein would be critical in the recognition of HCV 229E (although not necessarily in the recognition of FIPV). Thus, to determine whether the homologous fAPN sequences can functionally replace the human sequence, we constructed a chimerical gene, AP34 (Fig. 3a). AP34 contains a DNA fragment encoding amino acids 135–297 of the fAPN molecule inserted into the genetic background of pAPN, thereby replacing amino acids 132–294 of pAPN. The chimerical cDNA clone AP34 was analysed in a transfection/infection experiment and found to be a functional receptor for HCV 229E (Fig. 3b). As positive and negative controls in this experiment, we included the analysis of cells transfected with four APN encoding plasmids, AP1, AP30, AP7 and AP32, which have been described previously (Delmas et al., 1994; Kolb et al., 1996) These results define the fAPN amino acid residues essential in mediating HCV 229E infection and show that they are located within the amino-terminal part of the molecule.

**Identification of residues critical for the HCV 229E receptor function of hAPN**

When the relevant regions of the human, feline and porcine APN proteins are aligned it becomes evident that a stretch of approximately eight amino acids (located, for example, at amino acids 288–295 in the hAPN sequence) are particularly
A. F. Kolb, A. Hegyi and S. G. Siddell

Fig. 4. Alignment of human, feline and porcine APN proteins. Human APN amino acids 260–353 were aligned with the corresponding sequences from feline and porcine APN by using the Clustal method of the MegAlign program of the Lasergene Navigator analysis program (DNAstar, Madison, USA). Amino acids differing from the hAPN sequence are boxed.

(\textit{a})

<table>
<thead>
<tr>
<th>construct</th>
<th>susceptibility to infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAPN</td>
<td>+</td>
</tr>
<tr>
<td>pAPN</td>
<td>-</td>
</tr>
<tr>
<td>QSVNETAQ -&gt; DYVEKQAS</td>
<td>+</td>
</tr>
</tbody>
</table>

AP35

<table>
<thead>
<tr>
<th>+ TGEV</th>
<th>+ HCV 229E</th>
</tr>
</thead>
<tbody>
<tr>
<td>kDa</td>
<td></td>
</tr>
<tr>
<td>116</td>
<td>85</td>
</tr>
<tr>
<td>TGEV-N protein</td>
<td>HCV 229E-N protein</td>
</tr>
</tbody>
</table>

Fig. 5. (\textit{a}) Schematic representation of the hAPN, pAPN and the chimeric protein AP35. Human APN and pAPN sequences are represented by shaded and open boxes, respectively. The position of the PCR-directed mutation in AP35 is indicated. The susceptibility of transfected 293 HEK cells to infection with HCV 229E and TGEV is indicated. (\textit{b}) Western blot analysis of HCV 229E- and TGEV-infected cells. 293 HEK cells were transfected with the expression vectors AP1 and AP35 and challenged with HCV 229E or TGEV at an m.o.i. of 5. De novo synthesized nucleocapsid protein was detected in a Western blot by using the monoclonal antibodies NG12 and 3C/E4. The molecular masses of the marker proteins (Pharmacia) and the position of the nucleocapsid proteins (N) are indicated.

divergent (Fig. 4). It is also clear that, in this region, there is more difference between the hAPN and pAPN proteins than between the hAPN and fAPN proteins. In the hAPN molecule, this region is predicted to have a high hydrophilicity, a high surface probability and to fold into an alpha-chain (Kolb et al., 1996) and, taking all the data together, it seems very probable that these residues play a critical role in the hAPN–HCV 229E surface glycoprotein interaction.

In order to test this hypothesis, we introduced a variety of point mutations into the corresponding region of the pAPN gene. One set of mutations converted the sequence QSVNETAQ (pAPN amino acids 283–290) into DYVEKQAS...
(hAPN amino acids 288–295) (Fig. 5a). This construct, AP35, was then transfected into 293 HEK cells and challenged with HCV 229E or TGEV. As a control for this experiment, we included the construct AP1, i.e. wild-type pAPN. The results, shown in Fig. 5(b), demonstrate that the pAPN molecule containing this sequence alteration was converted into a functional receptor for HCV 229E and also retained its function as a TGEV receptor. This result defines a stretch of only eight amino acids that are critical for the HCV 229E receptor function of hAPN.

**Discussion**

The results presented in this study lead us to a number of important conclusions. First, we have identified a region within the fAPN molecule (amino acids 135–297) required for its function as a receptor for HCV 229E. As in the hAPN protein (Kolb et al., 1996), the amino acid sequences required for the HCV 229E receptor function of fAPN are located within the amino-terminal part of the protein. HCV 229E thus appears to be distinct from TGEV and CCV, which interact with amino acid sequences within the carboxyl-terminal part of the porcine and canine APN (cAPN) proteins, respectively (Benbacer et al., 1997). TGEV, CCV and FIPV are very closely related antigenically (Pedersen et al., 1978; Horzinek et al., 1982), whereas HCV 229E does not cross-react with antibodies raised against TGEV (Sanchez et al., 1990). The recognition of different determinants of the APN protein may, therefore, reflect both functional and structural differences between the HCV 229E surface glycoprotein and the surface glycoproteins of other group 1 coronaviruses.

Second, comparison of the relevant fAPN, hAPN and pAPN sequences allowed us to predict a short stretch of amino acids that were likely to be critical for the interaction between hAPN and HCV 229E. By PCR-directed mutagenesis, we converted this stretch of amino acids within the pAPN molecule to the corresponding residues of the hAPN molecule. The six residues which were mutated from the porcine to the human APN sequence were sufficient to convert the chimerical APN into a functional receptor for HCV 229E. This indicates that these residues are critically involved in the interaction of the HCV 229E surface glycoprotein with APN. Further mutations exchanging only three or two amino acids from pAPN to the corresponding hAPN residues did not produce functional HCV 229E receptors (data not shown) indicating, perhaps, that the eight amino acids may constitute a ‘linear’ epitope which has to be present to facilitate the HCV 229E receptor function. It is also worth noting that the amino acid sequence alterations introduced into the pAPN protein in the expression vector AP35 did not influence its ability to act as a receptor for TGEV. This suggests that the same region is not critically involved in the TGEV–pAPN interaction and may point to subtle differences in the biochemical interaction of TGEV and HCV 229E with their cognate receptors.

In the absence of any structural model for APN, it is difficult to speculate whether the hAPN region we have identified in this study interacts directly with the HCV 229E surface glycoprotein or whether it has an indirect, albeit essential, effect on the virus–receptor interaction. Similarly, we are not able to generally predict whether the APN regions involved in the interaction with HCV 229E and TGEV are truly independent (insofar as any protein ‘domain’ is independent of the total structure) or whether they constitute a complex ‘conformational’ epitope. However, we do believe that the data presented here can be used to obtain a better understanding of the HCV 229E–hAPN interaction and may, in the long-term, pave the way for the rational design of prophylactic or therapeutic compounds.

We are grateful to Drs Luis Enjuanes (Centro Nacional de Biotecnologia, Madrid, Spain), Raoul de Groot (University of Utrecht, The Netherlands) and Georg Herrler (Institute of Virology, University of Marburg, Germany) for providing valuable reagents.

**References**


Received 2 May 1997; Accepted 14 July 1997