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A Single Point Mutation of the Influenza C Virus Glycoprotein (HEF) Changes the Viral Receptor-Binding Activity

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From strain JHB/1/66 of influenza C virus a mutant was derived with a change in the cell tropism. The mutant was able to grow in a subline of Madin-Darby canine kidney cells (MDCK II) which is resistant to infection by the parent virus due to a lack of receptors. Inactivation of cellular receptors by either neuraminidase or acetyesterase and generation of receptors by resialylation of cells with N-acetyl-9-O-acetyleneuraminic acid (Neu5,9Ac2) indicated that 9-O-acetylated sialic acid is a receptor determinant for both parent and mutant virus. However, the mutant required less Neu5,9Ac2 on the cell surface for virus attachment than the parent virus. The increased binding efficiency enabled the mutant to infect cells with a low content of 9-O-acetylated sialic acid which were resistant to the parent virus. By comparing the nucleotide sequences of the glycoprotein (HEF) gene of the parent and the mutant virus only a single point mutation could be identified on the mutant gene. This mutation at nucleotide position 872 causes an amino acid exchange from threonine to isoleucine at position 284 on the amino acid sequence. Sequence similarity with a stretch of amino acids involved in the receptor-binding pocket of the influenza A hemagglutinin suggests that the mutation site on the influenza C glycoprotein (HEF) is part of the receptor-binding site.

INTRODUCTION

Influenza C viruses contain only one type of surface glycoprotein which is designated HEF. This protein is unique among myxovirus glycoproteins, because it has three activities: receptor binding, receptor destroying, and fusion (Ohuchi et al., 1982; Kitame et al., 1982; Vlasak et al., 1987; Herrler et al., 1988a; Formanowski and Meier-Ewert, 1988). The functional protein is a trimer made up of identical monomers which are encoded by the viral RNA segment 4.

The fusion activity requires the proteolytic cleavage of the precursor HEF, into subunits HEF, and HEF, (Kitame et al., 1982; Ohuchi et al., 1982). By analogy to influenza A and B viruses as well as paramyxoviruses a stretch of hydrophobic amino acids at the N-terminus of HEF, is assumed to be responsible for the fusion activity (Gething et al., 1978; Herrler et al., 1981; Sato et al., 1983; Nakada et al., 1984; Pfeifer and Compans, 1984).

The receptor-destroying activity of influenza C virus has been identified as an acetyesterase which releases the C-acetyl residue from N-acetyl-9-O-acetyleneuraminic acid (Neu5,9Ac2) (Herrler et al., 1985b; Schauer et al., 1988). It belongs to the class of serine hydrolases which are inactivated by diisopropylfluorophosphate (DFP) treatment (Muchmore and Varki, 1987). Amino acid 71 has been identified as the active-site serine (Herrler et al., 1988b; Vlasak et al., 1989).

Attachment of influenza C virus to cells has been shown to be mediated by Neu5,9Ac2-containing surface receptors (Rogers et al., 1986; Herrler and Klenk, 1987). Very little is known about the structure and the location of the receptor-binding site of influenza C viruses. Inhibition studies with DFP indicated that the active site of the esterase and the receptor-binding site are located on different epitopes on the glycoprotein (Muchmore and Varki, 1987). The epitopes may, however, be in close proximity to each other as indicated by analyses with monoclonal antibodies (Hachinohe et al., 1989).

MDCK II cells, a subline of Madin-Darby canine kidney cells, are resistant to infection by strain JHB/1/66 of influenza C virus due to a lack of receptors on the cell surface (Herrler and Klenk, 1987). From this strain a mutant was derived, which is able to grow in MDCK II cells (Szepanski et al., 1989). Here we show that the mutant has an increased efficiency in recognizing Neu5,9Ac2-containing receptors. This change, which also affects the cell tropism, is due to a single point mutation on the HEF gene.

MATERIALS AND METHODS

Cells

MDCK I and MDCK II cells were grown in 145-mm petri dishes (Greiner, FRG) in MEM containing 10% FCS.
Viruses

Influenza C virus, strain Johannesburg/1/66 (parent), and the mutant virus (mutant) were grown in MDCK I cells or embryonated eggs as described previously (Herrler and Klenk, 1987). Viruses were 35S-labeled by incubating the infected MDCK I cells in MEM containing [35S]methionine at a concentration of 5 μCi/ml (specific activity 1000 Ci/mmol; Amersham Buchler, UK). Virus was harvested 2 days p.i. and purified as described (Herrler et al., 1985a).

Glycoprotein isolation

Purified virus was incubated with a final concentration of 1% n-octyl glucopyranoside (Sigma) in 50 mM HEPES (pH 7.2), 100 mM NaCl, 10 mM CaCl2, and incubated for 10 min at room temperature. The samples were centrifuged for 10 min at 15,000 g and the supernatant was loaded on a 10-30% sucrose/HEPES/NaCl/CaCl2 gradient, containing 1% n-octyl glucopyranoside. After centrifugation at 214,000 g for 16 hr, fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing HEF were dialyzed and used for esterase assays.

Specific enzyme activity

The protein content of the HEF-containing fractions was measured using a commercial kit (Micro BCA, Pierce). The esterase activity was determined by incubation of influenza C glycoprotein with paranitrophenyl acetate (pNPA) at room temperature (Vlasak et al., 1987, Schauer et al., 1988). Alternatively, bovine submaxillary mucin (BSM, 2 mg/ml) was incubated with enzyme at 37°C for the times indicated. The release of acetate by the esterase was measured using a commercial kit (Boehringer, FRG). One unit is defined as the amount of esterase required to release 1 μmol acetate in 1 min from BSM (37°C) or pNPA (room temperature).

Treatment of cells with neuraminidase and acetyltransferase

MDCK cells were grown to confluency in 35-mm plastic petri dishes (Greiner). Confluent monolayers were incubated with 100 mU neuraminidase from C. perfringens (Sigma) for 30 min at 37°C. Cells were washed twice with PBS. To each sample 1 mU sialyltransferase and CMP-Neu5,9Ac2 at the concentrations indicated were added. After 1 hr at 37°C, cells were infected with parent or mutant virus. Two days after infection the yield of virus released into the medium was determined by HA-titration with chicken erythrocytes as described previously (Herrler et al., 1985c).

vRNA extraction

The virus suspension was incubated with 10% 1 M Tris–HCl (pH 7.4), 5% 1 M NaCl, 2% 0.5 M EDTA (pH 8.0), 0.8% SDS, and proteinase K at a final concentration of 200 μg/ml (Boehringer) for 2 hr at 37°C. RNA was purified by phenol/chloroform extraction and precipitated with 3 M sodium acetate/ethanol (Sambrook et al., 1989).

Amplification of viral sequences by PCR

cDNA was synthesized in a final reaction volume of 50 μl, containing 20 μl PCR buffer (10-fold), 14 μl dNTPs (125 mM each), 20 pmol primer, and 5 μg vRNA. The mixture was heated to 95°C for 1 min and afterward placed on ice for 10 min. Following the addi-
tion of 2 μl of reverse transcriptase from avian myeloblastosis virus (AMV-RT, Boehringer) and 2 μl RNAsin (Boehringer) the sample was incubated for 1 hr at 37°. The primer used was complementary to nucleotides 28–42 of the viral RNA segment 4.

dsDNA was amplified according to the instructions provided by the supplier of the reagents (Gene-Amp kit, TaqPolymerase 2, Cetus, Perkin–Elmer). The amplification reaction took place in a total volume of 100 μl containing 10 μl cDNA mixture (see above), 14 μl dNTPs (125 mM each), 6 μl PCR-buffer (10-fold), 16 pmol primer complementary to the noncoding strand, and 20 pmol of the primer complementary to the coding strand. The mixture was heated to 97° for 5 min, transferred onto ice, and 2.5 units of Thermus aquaticus (Taq) DNA-polymerase was added. The mixture was overlayed with 100 μl of light mineral oil. The amplification reaction was carried out for 30 cycles, each consisting of 30 sec denaturing at 93°, annealing for 1 min at 41°, and 1.5 min at 72° for extension, followed by a single cycle at 72° for 5 min. After removal of the mineral oil and chloroform extraction, an additional ether extraction was done and the dsDNA clarified using Centricon G30-columns (Amicon). The primers used for amplification were complementary to nucleotides 28–42 of the noncoding strand and to nucleotides 1425–1405 of the coding strand.

Nucleotide sequencing

Sequencing was done by a modified dideoxy chain termination method (Sanger et al., 1977). The template used was either purified virion RNA or dsDNA obtained by PCR amplification. For sequencing of dsDNA a Sequenase kit was used (U.S. Biochemical Corp.). The primers used for vRNA sequencing were those described by Buonagurio et al. (1985), primers complementary to mRNA sense were synthesized according to published data (Pfeifer and Compans, 1984). Primers for vRNA and dsDNA sequencing were 15 nucleotides in length and were synthesized using a Bioscience award synthesizer. Primers were desalted using NAP-5 columns, Sephadex G-25 (Pharmacia), and were checked on a 20% urea/acrylamide gel.

Primers are listed according to their nucleotide position based on published data (Pfeifer and Compans, 1984).

vRNA: 28–42, 191–205, 368–682, 517–531, 679–693, 874–888, 943–964, 1092–1106, 1237–1251, 1422–1436, 1612–1626, 1714–1728, 1881–1895.

mRNA: 103 89, 141 127, 927 912.

Sequences were obtained by running 6% urea/acrylamide gels in a BRL sequencing apparatus Model S2.

RESULTS

We have previously shown that the mutant virus grows in MDCK II cells, whereas the parent virus does not because of a lack of receptors (Szepanski et al., 1989). This difference raises the question of whether the parent and the mutant virus use different receptors. To compare the cellular structures that are recognized by both viruses inactivation studies were performed (Fig. 1). The receptors for both parent and mutant virus could be inactivated by neuraminidase treatment. Using MDCK I cells, receptors for the parent virus are inactivated more rapidly than receptors for the mutant virus. The receptors on MDCK II cells for the mutant virus are inactivated at a similar rate as the receptors for the parent virus on MDCK I cells. A similar inactivation pattern was obtained when the cells were treated with acetylesterase (data not shown). These findings indicate that both viruses recognize Neu5,9Ac2 as a receptor determinant. However, there appear to exist quantitative differences in the amount of receptors present on MDCK I and MDCK II cells and also in the efficiency of the parent and mutant to recognize these receptors.

In order to compare the binding of parent and mutant virus to surface receptors both viruses were analyzed for their ability to agglutinate red blood cells which had been modified by resialylation to contain different amounts of Neu5,9Ac2 on their surface (Fig. 2). Erythrocytes from 1-day-old chickens were used, because—in contrast to cells from adult chickens—they are resistant to agglutination by influenza C virus due to a lack of Neu5,9Ac2 on their surface (Herrler et al.,
FIG. 2. Generation of receptors for parent and mutant virus on red blood cells. One-day-old chicken erythrocytes were resialylated using 2 mU of Galβ1,4GlcNAcα2,6-sialyltransferase per sample and activated sialic acid (CMP-Neu5,9Ac$_2$) at the concentrations indicated. Following resialylation, cells were used to determine the hemagglutination titer of parent and mutant virus. Filled bars, parent virus; crosshatched bars, mutant virus.

Erythrocytes were first incubated with neuraminidase to provide acceptor sites for exogenous sialic acid and then resialylated using sialyltransferase and different concentrations of activated substrate (CMP-Neu5,9Ac$_2$). The modified erythrocytes were used to determine the hemagglutination titers of the parent and mutant virus. The virus solutions were adjusted to give equal titers in hemagglutination assays with erythrocytes from adult chickens. Hemagglutination by the parent virus was detectable after treatment of the cells with 0.1 μM of activated substrate. The maximum titer was obtained at a concentration of about 1 μM CMP-Neu5,9Ac$_2$ (Fig. 2). Agglutination by the mutant virus was observed with cells treated with as little as 0.02 μM of activated substrate. The maximum hemagglutination titer for the mutant virus was obtained when 0.1 μM CMP-Neu5,9Ac$_2$ was used for the resialylation of erythrocytes (Fig. 2). Thus, the mutant virus requires less Neu5,9Ac$_2$ on the cell surface for hemagglutination than does the parent virus. This result suggests a higher affinity to Neu5,9Ac$_2$-containing receptors for the mutant compared to the parent virus.

The binding properties of parent and mutant viruses were further investigated in a binding assay with resialylated erythrocytes. Purified parent and mutant viruses, which were similar in their protein content as well as in their HA activity, were incubated with different quantities of cells on ice. After sedimentation of the erythrocytes, the amount of virus remaining in the supernatant was measured (Fig. 3). As indicated by HA titration, both viruses were efficiently adsorbed by cells which had been resialylated in the presence of 1 μM of CMP-Neu5,9Ac$_2$ (Fig. 3a). When erythrocytes which had been resialylated at limiting concentrations of CMP-Neu5,9Ac$_2$ (0.25 and 0.5 μM) were used only the mutant virus was completely removed from the supernatant, while a substantial amount of parent virus was not bound by the cells (Figs. 3b and 3c). Erythrocytes containing the lowest amount of 9-O-acetylated sialic acid on their surface (resialylation at a CMP-Neu5,9Ac$_2$ concentration of 0.1 μM) were still able to partially adsorb the mutant virus; the parent virus was unaffected by these cells (Fig. 3d). Similar differences in the binding properties of both viruses were observed when the binding of [35S]methionine-labeled virus was quantitated by scintillation counting (not shown). This result indicates that binding of the mutant virus to erythrocytes requires less Neu5,9Ac$_2$ on the cell surface than does attachment of the parent virus.

Next we were interested in determining whether the results obtained with erythrocytes are also relevant for receptors on cultured cells which are used by influenza C virus to initiate infection. For resialylation of cultured cells we used MDCK II cells. They lack receptors for parent influenza C virus and the receptors for the mutant are inactivated by the neuraminidase treatment,
which is preceding the resialylation reaction. As described above for erythrocytes, different amounts of 9-O-acetylated sialic acid were attached to the cell surface by incubation of MDCK II cells with sialyltransferase and different concentrations of activated substrate (CMP-Neu5,9Ac₂). The modified cells were analyzed for their susceptibility to infection by measuring the virus released into the medium with a hemagglutination assay. Infection by the parent virus was detected after treatment of MDCK II cells with 15 μM of CMP-Neu5,9Ac₂ (Fig. 4). Infection by the mutant virus could be demonstrated after treatment of the cells with as little as 0.5 μM of activated substrate. HA titers of >100 HAU/ml were obtained at CMP-Neu5,9Ac₂ concentrations of ≥2.5 μM (Fig. 4). In the case of the parent virus even a concentration as high as 50 μM was not sufficient for such a virus yield. The result from Fig. 4 indicates that infection by the mutant virus requires less Neu5,9Ac₂ on the cell surface than the parent infection does.

The resialylation experiments demonstrate a difference in the receptor-binding efficiency between parent and mutant virus. To determine whether there is also a difference in the receptor-destroying enzyme we compared the specific esterase activities of both viruses. The viral glycoproteins were isolated by treatment with the nonionic detergent n-octylglucopyranoside and purified by sucrose gradient centrifugation. The specific enzyme activities of parent and mutant glycoprotein were determined by two different assays. In the first enzyme assay the cleavage of the low molecular weight synthetic substrate paranitrophenyl acetate (pNPA) was monitored (Vlasak et al., 1987; Schauer et al., 1988). In the second assay BSM, a high-molecular weight substrate rich in Neu5,9Ac₂ was incubated with the viral enzyme and the acetate release was determined (Herrler et al., 1985b). Under both conditions no significant difference in the specific enzyme activity was observed between parent and mutant glycoprotein—30 U/mg in the case of pNPA and 20 U/mg in the case of BSM.

The results presented above indicate that the mutant virus is able to infect MDCK II cells due to an increased efficiency in recognizing surface receptors. We analyzed whether this difference between parent and mutant is also reflected in the infection of other cells. As shown in Table 1, 3 days after infection with the parental virus the virus yields from the different cell lines varied to a great extent ranging from very high (MDCK I) to undetectable (Vero, CV-1, BHK). Varying amounts of virus were also obtained from the different cell types following infection with the mutant virus. However, in all cases tested, infection by the mutant virus resulted in higher yields than infection by the parent virus. None of the cell types tested was completely resistant against infection by the mutant.

Our results indicate that the viral surface glycoprotein (HEF) of the mutant virus has a change in the receptor-binding activity. To determine the mutation responsible for this difference, the RNA segments encoding the HEF protein were sequenced for both viruses. Direct sequencing was performed using viral RNA as a template and specific primers according to published data (Buonagurio et al., 1985). Five nucleotide changes were identified with regard to the published sequence for the HEF protein of strain C/JHB/1/66 (Pfeifer and Compans, 1984). Four of the changes were found on both parent and mutant genes. The parent and the mutant sequence differed only by a single point mutation at nucleotide position 872. This nucleotide exchange (C → T) causes a change at position

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**TABLE 1**

| Virus yield (HAU/ml) | Parent | Mutant |
|---------------------|--------|--------|
| MDCK I              | 2048   | 2048   |
| MDCK II             | 2      | 1024   |
| Chick embryo cells  | 64     | 128    |
| I C-MK₂             | 8      | 128    |
| MDBK                | 2      | 32     |
| BHK                 | <2     | 32     |
| Vero                | <2     | 8      |
| CV-1                | <2     | 4      |

**Fig. 4.** Generation of receptors for the parent and mutant virus on cultured cells. Cells were resialylated by incubation with 1 μlU of Galβ1,4GlcNAc a2,6-sialyltransferase and CMP-Neu5,9Ac₂ at the concentrations indicated for 1 hr at 37°C. Following infection with mutant or parent virus, the virus yield was determined 24 h.p.i. by HA titration of the supernatant. Filled bars, parent virus; crosshatched bars, mutant virus.


### TABLE 2

**Difference in the HEF Sequence of Parent and Mutant Virus of C/JHB/1/66 and Comparison of Partial Sequences of the Influenza C Glycoprotein and the Influenza A Hemagglutinin of Different Subtypes**

| Influenza subtypes | As No. | Sequence | AS No. |
|--------------------|--------|----------|--------|
| A/H1               | 220    | --R P K V K D Q H G R M N-- |        |
| A/H3               | 274    | --V S P Y T G N S G D T P-- |        |
| A/H5               | 231    | --R P W V R G L S S R I S-- |        |
| A/H6               | 284    | --R P Q I N G Q S G R I D-- | 205    |
| A/H7               |        | --R P Q I N G Q S G R I D-- |        |
| A/H10              |        | --R P Q V N G Q S G R I D-- |        |
| C/1/66 (parent)    |        | --V S P Y T G N S G D T P-- |        |
| C/1/66 (mutant)    |        | --V S P Y T G N S G D I P-- |        |

*Note.* The amino acid numbering of the H3 hemagglutinin has been used for all influenza A subtypes (Wilson et al., 1981). The aligning of the hemagglutinin sequences is adapted from Feldmann et al. (1988). The amino acids of HEF are numbered according to Pfeifer and Compans (1984).

284 in the amino acid sequence from threonine to isoleucine (Table 2). The sequence data were confirmed by sequencing of DNA segments obtained by the polymerase chain reaction (PCR). These results indicate that a single point mutation in the HEF gene results in an increased receptor-binding efficiency and in an extended cell tropism.

**DISCUSSION**

The ability of the mutant to grow in a cell line which lacks receptors for the parent virus suggested that a change in the receptor-binding activity of the mutant was the reason for the change in the cell tropism. However, no difference could be found with regard to the major receptor determinant. The cellular receptors for both viruses were inactivated by neuraminidase and acetyylesterase treatment. Furthermore, when receptors were generated by transfer of Neu5,9Ac₂ to cell surface components, cells became susceptible to infection by parent and mutant virus. These results indicate that Neu5,9Ac₂ is a crucial receptor determinant for both viruses, parent and mutant. We conclude therefore that both MDCK I and MDCK II cells contain Neu5,9Ac₂, but only the mutant is able to use the 9-O-acetylated sialic acids present on MDCK II cells to initiate infection. There are two ways to explain the failure of the parent virus to infect MDCK II cells. One possibility is that parent and mutant virus recognize 9-O-acetylated sialic acid in a different structural context. The difference could concern either the linkage between Neu5,9Ac₂ and the neighboring sugar or the type of oligosaccharide containing the sialic acid molecule.

This explanation appears to be unlikely because in hemagglutination assays the parent virus recognizes Neu5,9Ac₂ in three different linkages (α₂₃Galβ₁,₃GalNAc, α₂₃Galβ₁,₄GlcNAc, and α₂₆Galβ₁,₄GlcNAc) (Rogers et al., 1986). In addition, such diverse substances as rat α₁-macroglobulin, BSM, and bovine brain gangliosides may act as inhibitors for the parent and the mutant virus (Herrler et al., 1985a, b). These observations suggest that the sialic acid molecule itself is the crucial part of the receptor recognized by parent and mutant virus. The composition of the microenvironment appears to be of minor importance.

The differences between the parent and the mutant virus can be best accounted for if one assumes that the two viruses require different amounts of the receptors for binding to cells. This explanation is in accordance with the following experimental data: (i) the inactivation of the receptors for the parent virus by neuraminidase and acetyylesterase was more rapid than inactivation of the mutant receptors (Fig. 1); (ii) in the resialylation experiments, the mutant was more efficient in recognizing low amounts of 9-O-acetylated sialic acid (Figs. 2–4); and (iii) in hemagglutination inhibition tests, the mutant is more sensitive to rat serum and bovine submaxillary mucin than the parent virus (Szepanski et al., 1989). We conclude therefore that the mutant has a higher affinity for Neu5,9Ac₂ than the parent virus. This is also supported by the fact that the mutant has a growth advantage in all cell types tested. From several cell lines only low amounts of virus were obtained. Incorporation of Neu5,9Ac₂ into the plasma membrane resulted in an increase of the virus yield (Herrler and Klenk, 1987; Szepanski and Herrler, un-
published data); however, there were still major differences in the amount of virus released by the different cell types indicating that replication in some cells is restricted not only at the level of receptors. This is also suggested by the fact that our attempts to adapt influenza C virus to growth in Vero cells by serial passages were not successful.

From our results it can be predicted that MDCK II cells and other cell lines, which are resistant to infection by the parent virus but not by the mutant, contain less Neu5,9Ac2 than their surface than permissive cells such as MDCK I cells. This should be confirmed by chemical analysis in the future.

Comparison of the nucleotide sequences of the glycoprotein genes of parent and mutant virus indicates that a single point mutation is responsible for the change in the receptor-binding activity. Until now the location of the receptor binding site on the glycoprotein of influenza C virus is unknown. There are two possibilities how a point mutation might cause the change of the receptor-binding activity. The mutation may be located at or close to the receptor binding site and directly affect binding of the receptor. Another possibility is that the mutation leads to a conformational change of the glycoprotein and thereby indirectly changes the receptor-binding activity.

In two cases concerning the receptor-binding activity of the hemagglutinin of influenza A virus, it could be shown that a single point mutation may have a drastic effect on the receptor-binding activity. In one mutant an amino acid exchange at position 226 (Gln-Leu) changed the preference in the recognition of receptors from α2,3-linked sialic acid to α2,6-linked sialic acid (Rogers et al., 1983). A similar change in the binding specificity was reported for a mutant, in which tyrosine 205 was substituted for a serine (Suzuki et al., 1989). In the latter case, the mutation site was not in the receptor binding site; rather, it affected the receptor pocket of the neighboring hemagglutinin monomer.

The former mutation site (amino acid 226) has been shown to be part of the “left side” of the receptor pocket (Weis et al., 1988). In an alignment of the amino acid sequences of the hemagglutinin genes of six influenza A subtypes it is obvious that the sequence around amino acid 226 is not strictly conserved. However, within three subtypes the sequence -GQSG- is to be found (Table 2). A similar sequence, -GNSS-, differing only by a conservative amino acid exchange (asparagine for glutamine), is present also in the influenza C glycoprotein and is located close to the mutation site reported here (Table 2). This sequence similarity suggests that the mutation at amino acid 284 on the HLF glycoprotein may be located at or close to the receptor-binding site. If this is true, the mutation affects the receptor-binding activity directly rather than via a conformational change. This would be in accordance with the observation that the specific enzyme activity of the mutant is not affected by the mutation.

Whether we indeed obtained the first information about the location of the receptor-binding site on the influenza C glycoprotein has to be confirmed by site-directed mutagenesis and expression of the cloned gene in eukaryotic cells. For detailed information the three-dimensional structure has to be determined by X-ray crystallography. Preliminary results of such work have been reported recently at the International Congress of Virology (Rosenthal et al., 1990).

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