14. Look, A. T., Ashmun, R. A., Shapiro, L. H. & Peiper, S. C. *J. clin. Invest.* **83,** 1299–1307 (1989).
15. Kenny, A. J. & Maroux, S. *Physiol. Rev.* **62,** 91–128 (1982).
16. Haelterman, E. O., *J. Am. vet. med. Assoc.* **160,** 534–540 (1972).
17. Laude, H. *et al. J. gen. Virol.* **67,** 119–130 (1986).
18. Hauser, H., Howell, K., Dawson, R. M. C. & Bowyer, D. E. *Biochim. biophys. Acta* **602,** 567–577 (1980).
19. Sjöström, H. & Norén, O. *Eur. J. Biochem.* **122,** 245–250 (1982).
20. Johansen, T. H., Schøller, M. S., Tolstoy, S. & Schwartz, T. W. *FEBS Lett.* **267,** 289–294 (1990).
21. Kueng, W., Silber, E. & Eppenberger, U. *Analyt. Biochem.* **182,** 16–19 (1989).

ACKNOWLEDGEMENTS. We thank R. Christon for microvillar preparations, J. C. Huet for N-terminal sequencing, S. Maroux for anti-APN antibodies, and D. Rasschaert and M. Bremont for discussion. B.D. acknowledges the support of the Commission of the European Communities. L.K.V., H.S. and O.N. are members of the Biomembrane Research Center, Aarhus University and are supported by a grant from the Lundbeck foundation.

Human aminopeptidase N is a receptor for human coronavirus 229E

Curtis L. Yeager*, Richard A. Ashmun†‡, Richard K. Williams*, Christine B. Cardellichio*, Linda H. Shapiro†, A. Thomas Look†‡§ & Kathryn V. Holmes*‖

* Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799, USA
Departments of † Hematology/Oncology and ‡ Tumor Cell Biology, St Jude Children's Research Hospital, 332 North Lauderdale, Memphis, Tennessee 38105, USA
§ Department of Pediatrics, University of Tennessee College of Medicine, Memphis, Tennessee 38163, USA

HUMAN coronaviruses (HCV) in two serogroups represented by HCV-229E and HCV-OC43 are an important cause of upper respiratory tract infections[1]. Here we report that human aminopeptidase N, a cell-surface metalloprotease on intestinal, lung and kidney epithelial cells[2–5], is a receptor for human coronavirus strain HCV-229E, but not for HCV-OC43. A monoclonal antibody, RBS, blocked HCV-229E virus infection of human lung fibroblasts, immunoprecipitated aminopeptidase N and inhibited its enzymatic activity. HCV-229E-resistant murine fibroblasts became susceptible after transfection with complementary DNA encoding human aminopeptidase N. By contrast, infection of human cells with HCV-OC43 was not inhibited by antibody RBS and expression of aminopeptidase N did not enhance HCV-OC43 replication in mouse cells. A mutant aminopeptidase lacking the catalytic site of the enzyme did not bind HCV-229E or RBS and did not render murine cells susceptible to HCV-229E infection, suggesting that the virus-binding site may lie at or near the active site of the human aminopeptidase molecule.

To develop a monoclonal antibody against the HCV-229E receptor, we produced hybridomas against deoxycholate-solubilized membrane proteins of two HCV-229E-susceptible human cell lines (WI38 lung fibroblasts and HL60 myeloid leukaemia cells). A monoclonal antibody designated RBS protected WI38 and RD human cell lines from HCV-229E-induced cytopathic effects and protected WI38 cells from virus infection (Fig. 1*a–c*). RBS pretreatment reduced the number of HCV-229E-infected WI-38 cells at 10 h post-infection by 96%, compared with cells pretreated with control mouse ascites. By contrast, RBS did not inhibit replication of HCV-OC43 in WI38 or RD cells, indicating that the receptor specificities of HCV-OC43 and HCV-229E are different.

Susceptibility to HCV-229E infection in mouse–human somatic cell hybrids depends on a gene located on human chromosome 15 (ref. 6). A promising candidate for the HCV-229E receptor is human aminopeptidase N (hAPN; EC 3.4.11.2), a cell-surface glycoprotein encoded by a gene on bands q25–q26 of human chromosome 15 (ref. 7) and expressed on human lung, renal and intestinal epithelial cells, fibroblasts and nerve synapses[2–5]. This exopeptidase removes amino-terminal residues to complete the digestion of short peptides in the gut and helps break down neurotransmitter peptides in the brain[2,3,5,8]. hAPN is identical to CD13, a glycoprotein identified on granulocytes, monocytes and their bone marrow progenitors[9,10]. Porcine aminopeptidase N is a receptor for transmissible gastroenteritis virus, a porcine coronavirus in the same serogroup as HCV-229E (ref. 11). Because aminopeptidase from humans, pigs and other mammals are structurally similar[9,12–14], we investigated whether HCV-229E and RBS would bind specifically to hAPN and whether expression of hAPN by murine cells would make them susceptible to infection with HCV-229E.

Murine NIH3T3 cells transfected with hAPN cDNA in a retroviral vector[9] (hAPN-3T3) and untransfected NIH3T3 cells were challenged with HCV-229E and HCV-OC43 to determine their susceptibility to virus infection. Although the control NIH3T3 cells were resistant to HCV-229E infection (Fig. 1*d*), the hAPN-transfected mouse cells were susceptible to infection with this virus (Fig. 1*e*). By contrast, hAPN-3T3 cells were no more susceptible than NIH3T3 cells to infection with HCV-OC43 (data not shown). Thus, expression of hAPN confers HCV-229E susceptibility, but not HCV-OC43 susceptibility, on murine cells.

We analysed binding of RBS to membrane preparations from hAPN-3T3 or parental NIH3T3 fibroblasts. The antibody bound to membranes of hAPN-3T3 but not to those of NIH3T3 cells (Fig. 2*a*), indicating that RBS recognized hAPN. Similarly, HCV-229E virions bound more strongly to hAPN-3T3 membranes than to NIH3T3 membranes (Fig. 2*b*), and RBS competi-

TABLE 1 Biological activities of anti-aminopeptidase N monoclonal antibodies and aminopeptidase N inhibitors

	Inhibition of enzyme activity (%)*	Binding to $hAPN_{mut}$-3T3†	Inhibition of HCV-229E infection‡
Monoclonal antibodies			
WM15	91	−	+
RBS	90	−	+
MY7	42	+	+
Chemical inhibitors§			
Actinonin	100	NA	−
Bestatin	100	NA	−
1,10-Phenanthroline	100	NA	+
2,2′-Dipyridyl	100	NA	+

* The inhibition of hAPN activity was determined as described in the legend to Fig. 2*d*.

† Binding of antibodies to $hAPN_{mut}$-3T3 cells was measured by flow cytometry, as outlined in the legend to Fig. 3. The mutant lacks peptidase activity; thus, assays for chemical inhibition were not applicable (NA).

‡ Confluent monolayers of WI38 cells in 96-well plates were pretreated with dilutions of antibodies or inhibitors in medium for 1 h, and then challenged with 1×10^3 p.f.u. per well of HCV-229E. After 1 h of adsorption, the inoculum was removed, and the cells were incubated with fresh medium containing antibodies or inhibitors for 48 h, at which time the monolayers were examined for virus-induced cytopathic effects. Such effects were evident in HCV-229E-infected controls pretreated with normal serum, but not in mock-infected controls. Plus signs, HCV-229E-induced cytopathic effects were inhibited by antibodies up to a dilution of 1:200. All incubations were at 37 °C.

§ Inhibitors were tested at the following concentrations: bestatin, 1 mg ml^{-1}; 1,10-phenanthroline, 1.5 mM; 2,2′-dipyridyl, 2.5 mM; actinonin, 2.7 mM. Antibodies were tested at concentrations that saturated available binding sites in flow cytometric assays.

‖ To whom correspondence should be addressed.

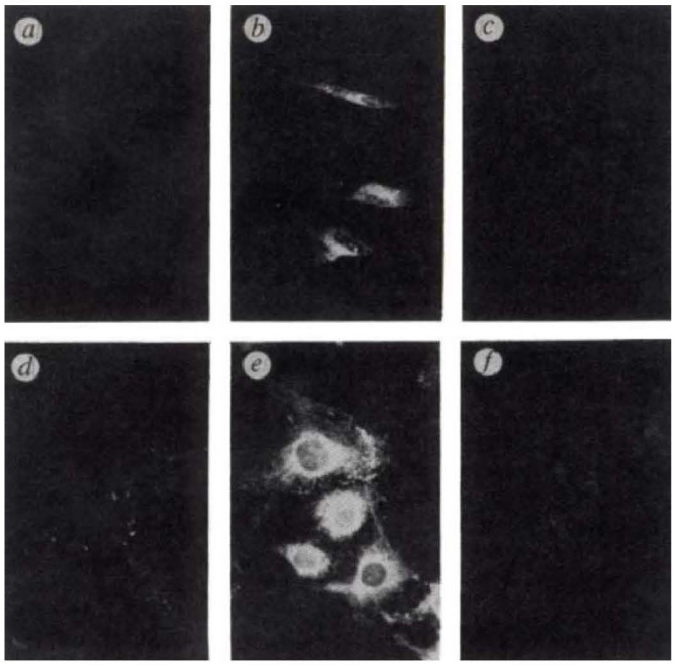

FIG. 1 Inhibition of HCV-229E replication in WI38 cells by anti-receptor monoclonal antibody RBS and susceptibility to HCV-229E of mouse cells expressing normal and mutant hAPN. *a*, Uninfected WI38 cells; *b*, WI38 cells infected with HCV-229E; or *c*, WI38 cells treated with RBS and then challenged with HCV-229E. Three types of murine NIH3T3 cells were challenged with HCV-229E: *d*, native cells; *e*, transformants expressing high levels of hAPN (hAPN-3T3)[7]; and *f*, cells engineered to express a mutant hAPN ($hAPN_{mut}3T3$)[11], which lacks 39 amino acids, including the active site of the enzyme.

METHODS. Hybridoma supernatants (1,624) were tested for the ability to inhibit HCV-299E infection of WI38 cells and one, RBS, was positive. The RBS hybridoma was subcloned three times, and ascites fluid produced by these cells in BALB/c mice was used as the source of antibody. Cells grown on coverslips were pretreated for 1 h at 37 °C with a 1:10 dilution of RBS, control ascites or control medium, and then challenged with 1×10^4 p.f.u. HCV-229E. Cells were incubated at 37 °C for 10 h (*a–c*) or for 18 h (*d–f*) and fixed in cold acetone. Antisera remained in the plates throughout the experiment. Intracellular HCV-229E antigens were detected with goat anti-HCV-229E and rhodamine-labelled rabbit anti-goat immunoglobulin.

tively inhibited virus binding to hAPN-3T3 membranes (Fig. 2*c*), suggesting that RBS and HCV-229E may recognize adjacent or overlapping epitopes of the peptidase. Some, but not all, antibodies that bind to extracellular epitopes of hAPN inhibit enzymatic activity[15]. We compared the ability of RBS to inhibit the enzymatic activity of hAPN with that of hAPN/CD13-specific antibodies MY7 and WM15. RBS blocked hAPN activity as efficiently as WM15 and better than MY7 (Fig. 2*d*).

Flow cytometric results indicated that both RBS and MY7 bound to hAPN-3T3 cells (Fig. 3*a*, *b*), but not to untransfected NIH3T3 cells. RBS also immunoprecipitated the mature 150K hAPN glycoprotein and its 130K intracellular precursor[16] from

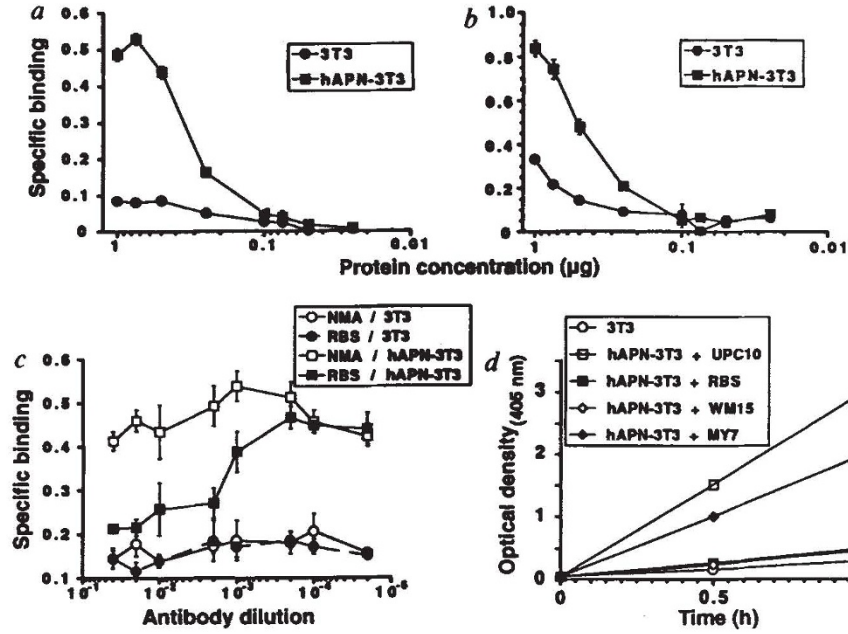

FIG. 2 Competitive binding of the RBS antibody and HCV-299E to hAPN. Native mouse NIH3T3 cells (3T3) or NIH3T3 transformants expressing high levels of hAPN (hAPN-3T3) were tested. Membrane preparations were used in ELISA assays to measure: *a*, RBS binding; *b*, HCV-229E binding; or *c*, competitive binding of RBS and HCV-229E. *d*, Aminopeptidase N enzymatic activity on the surface of intact cells in the presence of RBS, the hAPN-specific antibodies WM15 or MY7, or the mouse myeloma protein UPC10 (control).

METHODS. Cells were Dounce-homogenized and nuclei were removed by low-speed contrifugation. Cell lysates were pelleted by ultracentrifugation and the membranes resuspended in 10 mM Tris-EDTA with 1% aprotinin. Protein concentrations were determined by Bradford assay (Bio-Rad). Membrane preparations were serially diluted in carbonate buffer, pH 9.6, incubated overnight on Dynatech Immulon 1 microtitre plates, blocked with TBS-Tween buffer (0.15 M NaCl, 0.05 M Tris–HCl, 0.001 M EDTA, 0.05% Tween-20) containing 5% BSA. For detection of antibody binding, 100 μl of a 1:50 dilution of either RBS or normal mouse ascites in TBS-Tween buffer with 0.1% BSA was added to each well, and peroxidase-labelled goat anti-mouse immunoglobulin (Boehringer Mannheim) diluted 1:1,000 was used to detect bound antibody. For detection of HCV-229E binding, membrane-coated plates were incubated with dilutions of HCV-229E or control medium, followed by goat anti-HCV-229E, biotinylated rabbit anti-goat immunoglobulin and streptavidin–peroxidase (Kirkeguard and Perry). For competitive inhibition of HCV-229E binding by RBS, dilutions of RBS ascites in medium were incubated with membranes on plates before and after the addition of HCV-229E. Incubations were at room temperature for 1 h. Assays were completed by addition of TMB substrate (Kirkeguard and Perry) and read on a Dynatech laboratories MR700 ELISA Reader. Background values obtained with membrane preparations alone were subtracted to give values for specific binding.

For measurements of enzymatic activity on intact cells, 2×10^5 cells were washed in PBS, resuspended in isotonic defined medium (50 mM phosphate buffer, pH 7.4) and incubated at 37 °C for 1 h in an excess of RBS, WM15, MY7 or UPC10 (control mouse myeloma protein). After incubation, and while still at 37 °C, the APN substrate, alanine-*p*-nitroaniline, was added to a final concentration of 6 mM. Samples were periodically removed and chilled to 4 °C to arrest enzymatic activity. After centrifugation, free *p*-nitroaniline present in the cell-free supernatants was detected by absorbance measurement at 405 nm. Degradation of substrate in the absence of cells over the same time period was minimal. All measurements were made in triplicate.

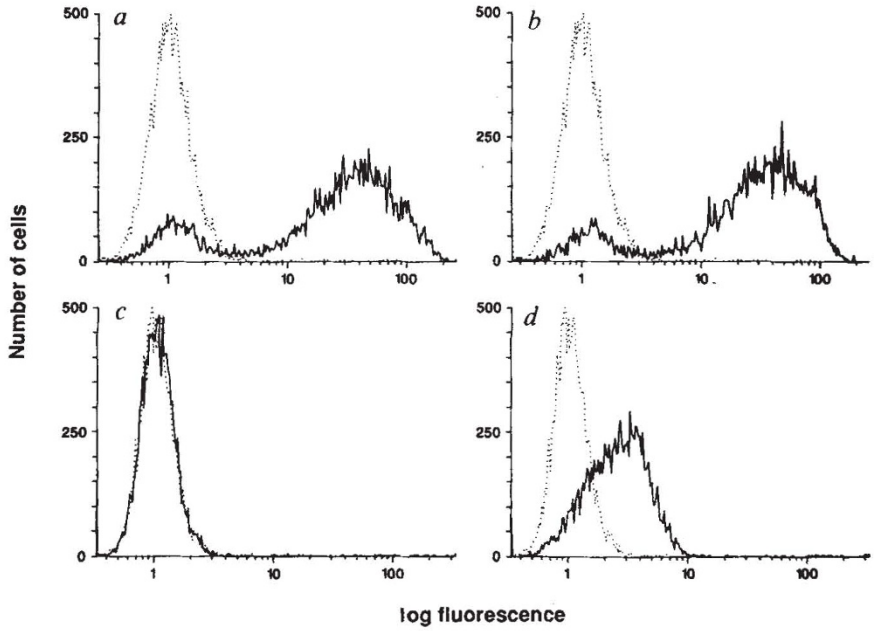

FIG. 3 Flow cytometric analysis of antibody binding to NIH3T3 transfectants. *a*, The hAPN-3T3 cells incubated with either the RBS antibody (solid line) or the control mouse myeloma protein UPC10 (dotted line). *b*, The hAPN-3T3 cells incubated with either the MY7 antibody (solid line) or UPC10 (dotted line). *c*, The $hAPN_{mut}$-3T3 cells incubated with either the RBS antibody (solid line) or UPC10 (dotted line). *d*, the $hAPN_{mut}$-3T3 cells incubated with either the MY7 antibody (solid line) or UPC10 (dotted line).
METHODS. Cells (1×10^6) were incubated at 4 °C for 30 min in a titred excess of RBS, MY7 (Coulter), or control mouse myeloma protein (UPC10; Organon-Teknika-Cappel). After being washed twice with cold staining medium (Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, L-glutamine, 10 mM HEPES, antibiotics and 2 mM sodium azide), the cells were incubated at 4 °C for 30 min in a titred excess of fluoresceinated, affinity-purified goat antiserum to mouse immunoglobulins (Coulter). Washed cells were resuspended in cold staining medium containing 0.25 mM propidium iodide and those that excluded the dye were analysed by flow cytometry.

hAPN-3T3 cells (data not shown). RBS did not bind to NIH3T3 cells transfected with a cDNA encoding a mutant hAPN glycoprotein ($hAPN_{mut}-3T3$; Fig. 3*c*)[17] lacking a 39-amino-acid region (amino acids 360–398) which includes the His-Glu-X-X-His sequence that mediates zinc binding and catalytic activity in a series of related metalloproteases[18]. MY7 bound to $hAPN_{mut}$-3T3 cells (Fig. 3*d*), confirming expression of the mutant protein on the cell surface. But $hAPN_{mut}$-3T3 cells were not susceptible to HCV-229E infection (Fig. 1*f*), indicating that both RBS and HCV-229E recognize epitopes that were eliminated or altered in the mutant polypeptide.

We investigated whether the ability of antibodies to block HCV-229E infectivity correlated with their ability to block enzymatic activity or to bind to the mutant hAPN polypeptide. RBS and WM15 both blocked enzyme activity and virus infection, but failed to bind to $hAPN_{mut}$-3T3 cells (Table 1). By contrast, although MY7 also inhibited HCV-229E infection, it only partially inhibited enzyme activity and recognized the mutant hAPN glycoprotein. Infection of WI38 cells by HCV-229E was not prevented by either actinonin or bestatin, small inhibitory molecules that bind competitively to the active site of hAPN[19–21]. By contrast, HCV-229E infection was inhibited by 1,10-phenanthroline and 2,2′-dipyridyl, which block aminopeptidase N activity by chelating the zinc required by enzymatically active hAPN molecules[18]. These chelators may alter the conformation of hAPN epitopes, preventing virus attachment. Other compounds that prevent binding of HCV-229E to hAPN may prove useful as drugs against infection with HCV-229E and related coronaviruses.

We have shown that human coronavirus HCV-229E uses human aminopeptidase N as a receptor and that antibodies directed against the active site of this enzyme prevent virus infection of human cells. A serologically unrelated human respiratory coronavirus, HCV-OC43, does not use hAPN as a receptor, and its receptor is unknown. HCV-OC43 is related to murine coronavirus MHV-A59, whose receptor[22–24] is a member of the carcinoembryonic antigen family of glycoproteins in the immunoglobulin superfamily. Thus, two related coronaviruses, HCV-229E and TGEV, use aminopeptidase N glycoproteins of their normal host species as receptors, whereas two coronaviruses in a different serogroup do not use this aminopeptidase as their receptors. Possibly, coronaviruses related to HCV-229E, such as canine or feline coronaviruses, also use as receptors aminopeptidase or related metalloproteases, such as aminopeptidase A (refs 25, 26) or neutral endopeptidase[26–30]. Because of their abundance at the apical surfaces of epithelial cells in the alimentary and respiratory tracts, these metalloproteases are promising candidates for receptors of other groups of mammalian viruses or for bacteria or fungi. □

Received 3 February; accepted 2 April 1992.

1. McIntosh, K. in *Virology* 2nd edn (eds Fields, D. N. *et al.*) 857–864 (Raven, New York, 1990).
2. Noren, O., Sjostrom, H., Danielsen, E. M., Cowell, G. M. & Skovbjerg, H. in *Molecular and Cellular Basis of Digestion* (ed. Desnuelle, P.) 335–363 (Elsevier, Amsterdam, 1986).
3. Semenza, G. *A. Rev. Cell Biol.* **2,** 255–313 (1986).
4. Kenny, A. J. & Maroux, S. *Physiol. Rev.* **62,** 91–128 (1982).
5. Matsas, R., Stephenson, S. L., Hryszko, J., Kenny, A. J. & Turnover, A. J. *Biochem. J.* **231,** 445–449 (1985).
6. Sakaguchi, A. Y. & Shows, T. B. *Somatic Cell Genet.* **8,** 83–94 (1982).
7. Look, A. T. *et al. J. clin. Invest.*, **78,** 914–921 (1986).
8. Turner, A. J., Hooper, N. M. & Kenny, A. J. in *Mammalian Ectoenzymes* (eds Kenny, A. J. & Turner, A. J.) 211–248 (Elsevier, Amsterdam, 1987).
9. Look, A. T., Ashmun, R. A., Shapiro, L. H. & Peiper, S. C. *J. clin. Invest.* **83,** 1299–1307 (1989).
10. Look, A. T. *et al.* in *Leukocyte Typing IV* (eds Knapp, W. *et al.*) 784–787 (Oxford Univ. Press. Oxford, 1989).
11. Delmas, B. *et al. Nature* **357,** 417–420 (1992).
12. Olsen, J., Sjqstrom, H. & Noren, O. *FEBS Lett.* **251,** 275–81 (1989).
13. Noren, O. *et al. FEBS Lett.* **259,** 107–112 (1989).
14. Watt, V. M. & Yip, C. C. *J. biol. Chem.* **264,** 5480–5487 (1989).
15. Ashmun, R. A. & Look, A. T. *Blood* **75,** 462–469 (1990).
16. Look, A. T. *et al. J. clin. Invest.* **75,** 569–579 (1985).
17. Ashmun, R. A., Shapiro, L. H. & Look, A. T. *Blood* (in the press).
18. Vallee, B. L. & Auld, D. S. *Biochemistry* **29,** 5647–5659 (1990).
19. Umezawa, H. *et al. J. Antibiotics* **38,** 1629–1630 (1985).
20. Rich, D. H., Moon, B. J. & Harbeson, S. *J. med. Chem.* **27,** 417–422 (1984).
21. Wilkes, S. H. & Prescott, J. M. *J. biol. Chem.* **260,** 13154–13162 (1985).
22. Williams, R. K., Jiang, G. S., Snyder, S. W., Frana, M. F. & Holmes, K. V. *J. Virol.* **64,** 3817–3823 (1990).
23. Williams, R. K., Jiang, G. S. & Holmes, K. V. *Proc. natn. Acad. Sci. U.S.A.* **88,** 5533–5536 (1990).
24. Dveksler, G. S. *et al. J. Virol.* **65,** 6881–6891 (1991).
25. Wu, Q., Lahti, J. M., Air, G. M., Burrows, P. D. & Cooper, M. D. *Proc. natn. Acad. Sci. U.S.A.* **87,** 993–997 (1990).
26. Wu, Q., Li, L., Cooper, M. D., Pierres, M. & Gorvel, J. P. *Proc. natn. Acad. Sci. U.S.A.* **88,** 676–680 (1991).
27. Devault, A. *et al. EMBO J* **6,** 1317–1322 (1987).
28. Malfroy, B., Kuang, W.-J., Seeburg, P. H., Mason, A. J. & Schofield, P. R. *FEBS Lett.* **229,** 206–210 (1988).
29. Letarte, M. *et al. J. exp. Med.* **168,** 1247–1253 (1988).
30. Shipp, M. A. *et al. Proc. natn. Acad. Sci. U.S.A.* **86,** 297–301 (1989).

ACKNOWLEDGEMENTS. We thank H. Nguyen, E. Wingfield, E. Mann and K. Coleman for technical assistance, D. Snyder, C. Dieffenbach, G. Dveksler and D. Wessner for discussions, and J. Gilbert for review.