Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3855–3858, May 1992
Microbiology

Human linear B-cell epitopes encoded by the hepatitis E virus include determinants in the RNA-dependent RNA polymerase

(hepatitis E virus/B cell epitopes/synthetic peptides/RNA-dependent RNA polymerase/non-structural proteins)

M. KAUR*, K. C. HYAMS*, M. A. PURDY†, K. KRAWCZYNSKI†, W. M. CHING*, K. E. FRY‡, G. R. REYES‡, D. W. BRADLEY†, AND M. CARL*§

*Accelerated Product Development and Infectious Disease Threat Assessment, Naval Medical Research Institute, Bethesda, MD 20814-5055; †Hepatitis Branch, Division of Viral Diseases, Centers for Disease Control, Atlanta, GA 30333; and ‡Molecular Virology Department, Genelabs Incorporated, Redwood City, CA 94063

Communicated by J. H. S. Gear, January 15, 1992

ABSTRACT **Hepatitis E virus is responsible for both sporadic and epidemic hepatitis in developing countries. The nonenveloped virus is 27–34 nm in diameter and has been shown to contain a single-strand, positive-sense, polyadenylylated RNA genome of ≈7.5 kilobases. The nucleotide sequence of the Burma strain of hepatitis E virus has been reported and three open reading frames (ORFs) have been identified. The deduced amino acid sequence from each of these ORFs was used to synthesize overlapping peptides (decamers overlapping at every fourth amino acid) on a solid phase. These peptides were then tested in an ELISA with pooled acute-phase sera from known cases of enterically transmitted non-A, non-B hepatitis collected in the Sudan. Linear B-cell epitopes were identified in all three ORFs. Epitopes were identified throughout the polyprotein encoded by ORF1, but they appeared to be particularly concentrated in the region of the RNA-dependent RNA polymerase. Distinct epitopes were identified in the presumed structural protein encoded by ORF2, and one epitope was identified close to the carboxyl terminus of the protein encoded by ORF3. These data precisely pinpoint linear B-cell epitopes recognized by antibodies from patients with acute hepatitis E and identify an antibody response directed against the RNA-dependent RNA polymerase.**

A major form of non-A, non-B hepatitis is the enterically transmitted (ET-NANB) form that is known to frequently occur in developing countries. Outbreaks of ET-NANB hepatitis in Asia (1–3), USSR (4), Costa Rica and Mexico (5), and several African nations (6) have generally been traced to contaminated water (7, 8). Males and females appear to be equally affected, and the clinical course is similar to that of classic hepatitis A except for a high mortality rate (10–20%) in pregnant women (9, 10). There is no evidence of chronic hepatitis following acute ET-NANB hepatitis (11).

Virus-like particles with a diameter of 27–34 nm have been recovered from the stools of patients with ET-NANB hepatitis (10–12). The viral agent has been serially transmitted in cynomolgus macaques (13–15), and a partial cDNA clone from the responsible virus was isolated from infected cynomolgus bile (15). The hepatitis E virus (HEV) appears to be distantly related to known positive-strand RNA viruses (16) and has a genome of ≈7.5 kilobases (kb) that contains three open reading frames (ORFs) encoding viral proteins (17). ORF1, the largest of the three, extends ≈5 kb from the 5′ end. ORF2, the next largest, begins 37 base pairs (bp) downstream of ORF1 and extends ≈2 kb to the termination codon present 65 bp from the 3′-terminal stretch of adenosine residues. ORF3 partially overlaps ORF1 and ORF2 and contains only 369 bp (17). ORF1 is believed to encode nonstructural proteins including an RNA-dependent RNA polymerase (RDRP) (15, 16) and a helicase-like region believed to promote the unwinding of DNA·RNA duplexes, which are required for genome replication, recombination, repair, and transcription of viral genes (16). ORF2 encodes a protein with a signal peptide at its amino terminus and a capsid-like protein region with a high content of basic amino acids (17). ORF3 overlaps both ORF1 and ORF2, and its expression as well as that of ORF2 presumably involves subgenomic transcripts that are coterminal with the 3′ end of the virus (17, 18).

To identify immunologically relevant epitopes of HEV, we synthesized overlapping peptides on a solid phase, based on the deduced amino acid sequences of the three ORFs of HEV (Burma; B) (17). The peptides were then tested for reactivity with sera derived from documented cases of acute hepatitis E from the Sudan (19). The present study demonstrates that the human B-cell response to HEV in acutely infected humans is directed against both nonstructural and structural proteins of the virus and includes a prominent response directed against the RDRP. The precisely identified epitopes may be important in designing serological tests for diagnosing acute infection and might represent determinants that are critical for monitoring the immunologic response and outcome of infection with HEV.

MATERIALS AND METHODS

Synthesis of Overlapping Peptides. The method of Geysen *et al.* (20) was used in this synthesis. Briefly, decamers overlapping by every four amino acids were synthesized on polypropylene pin heads (Cambridge Research Biochemicals, Northwich, U.K.). All peptides were synthesized in duplicate. The amino acids used were pentafluorophenyl esters of 9-fluorenylmethoxycarbonyl derivatives. The blocks, each containing 96 polypropylene pin heads, were immersed in dimethylformamide (DMF) (Baxter Healthcare, Burdick and Jackson Div., Muskegon, MI) for 5 min followed by deprotection in 20% piperidine in DMF for an hour. The blocks were then washed in 1% acetic acid (Mallinckrodt) in DMF for 5 min followed by a similar wash in DMF. After four washes in methanol, the blocks were air-dried for an hour. Prior to the addition of amino acids at a concentration of 30 mM, the blocks were allowed to sit in DMF for 5 min. The amino acid derivatives were dissolved in an equimolar solution of 1-hydroxybenzotriazole (Sigma) in DMF. One hundred microliters of the desired amino acids was dispensed into appropriate wells of polypropylene microtiter plates, which were then incubated overnight at room temperature.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ORF, open reading frame; ET-NANB hepatitis, enterically transmitted non-A, non-B hepatitis; HEV, hepatitis E virus; RDRP, RNA-dependent RNA polymerase.

§To whom reprint requests should be addressed.

The following day, after the blocks were washed once in DMF and four times in methanol and air-dried as previously, deprotection was performed. The above steps were repeated until the desired number of amino acids had been added. The N-terminal amino group was then acetylated in DMF containing 10% acetic anhydride (Sigma) and 2% diisopropylethylamine (Sigma). After an incubation of 90 min at room temperature, the peptides were deblocked for 4 hr in trifluoroacetic acid (Sigma) containing 2.5% phenol (Sigma) and 2.5% 1,2-ethanedithiol (Sigma). This was followed by washes in dichloromethane (Aldrich) and in dichloromethane containing 5% diisopropylethylamine. The blocks were then air-dried and rinsed in distilled water and in methanol (Mallinckrodt). After air-drying, the blocks were stored in silica gel for at least 18 hr at room temperature prior to storage at −20°C. To monitor the synthesis, peptides with amino acid sequences PLAQ and GLAQ were simultaneously synthesized on control pins.

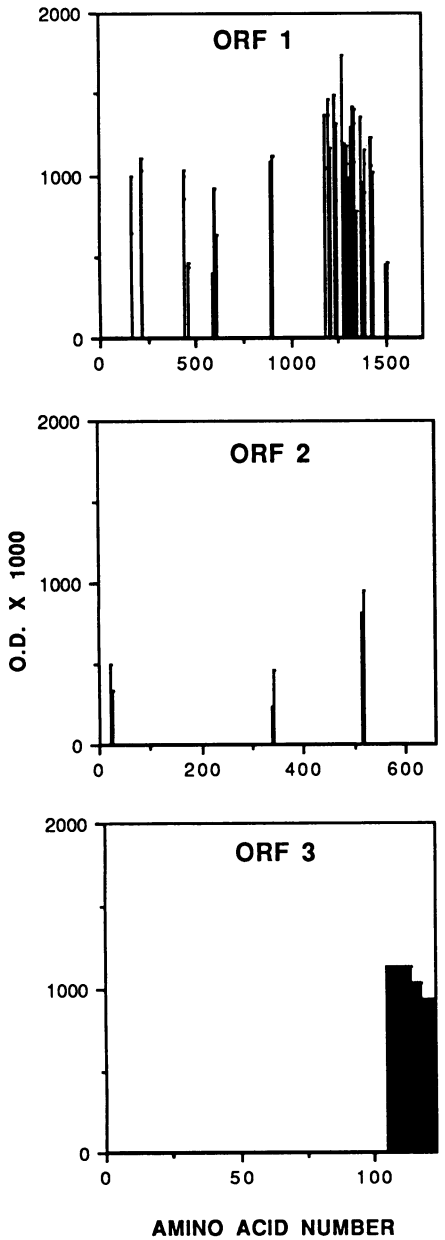

FIG. 1. Graphical representation of reactive epitopes encoded by the three ORFs. An epitope was considered reactive if (*i*) the optical density resulting from the interaction of pooled sera from patients with acute hepatitis E and peptide was ≥3-fold higher than the interaction of the same peptide with the pooled control sera and (*ii*) the peptide was adjacent to at least one other peptide that met the same criterion. ORF1 encodes a protein of 1693 amino acids, ORF2 encodes a protein of 660 amino acids, and ORF3 encodes a protein of 123 amino acids.

Human Sera. Serum samples from 11 Sudanese pediatric patients (14 years of age or less) with acute hepatitis E diagnosed by a Western blot assay (19) were pooled and tested for reactivity to the overlapping peptides described above. Serum samples from 11 control patients in the same study (19) with no evidence of acute hepatitis E (as determined by Western blot assay) were also pooled and served as a negative control.

Testing of Human Sera by ELISA. The blocks were allowed to reach ambient temperature before incubation for an hour at 37°C in blocking reagent [5% bovine serum albumin (Calbiochem), 0.05% Tween 20 (Sigma), and 0.05% sodium azide (Fisher) in phosphate-buffered saline (PBS, pH 7.2; GIBCO)]. The blocks were incubated overnight with the appropriate serum sample (1:100 dilution in blocking reagent) or with PBS at 4°C. All pooled sera were tested against the duplicate syntheses. The PLAQ and GLAQ pins were tested with commercially available murine sera (Cambridge Research Biochemicals).

The following day the blocks were washed four times in PBS (pH 7.2) containing 0.05% Tween 20 and 0.05% sodium azide (wash buffer) and incubated for an hour at 37°C with alkaline phosphatase labeled goat anti-human IgG + IgM at a 1:1000 dilution in the wash buffer. The PLAQ and GLAQ pins received a similarly labeled goat anti-mouse IgG at a 1:1000 dilution. The blocks were once again washed as described earlier and the color was developed by using a commercially available phosphatase substrate kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The intensity of the color reaction was determined on the UVmax (Molecular devices) at 405 nm. The readings obtained for peptides incubated with PBS were then deducted from the corresponding readings obtained with the serum samples.

Sonication of Blocks. After use, the blocks were washed in PBS for 5 min prior to sonication at 47 kHz and 60°C in sodium phosphate buffer (pH 7.2) containing 1% SDS (Sigma) and 0.1% 2-mercaptoethanol (Sigma) for 40 min. Sonication

Table 1. Protein regions of HEV recognized by antibodies from acute cases of ET-NANB hepatitis

Peptide	Amino acids position	Sequence
ORF1		
1	169–182	MSPSDVAEAMFRHG
2	221–234	YEGDTSAGYNHDVS
3	461–474	TAIRKALSKFCCFM
4	597–618	SQSTMAAGPFSLTYAASAAGLE
5	901–914	AWERNHRPGDELYL
6	1185–1198	DAPGLLREVGISDA
7	1205–1222	LAGGEIGHQRPSVIPRGN
8	1237–1254	CQISAFHQLAEELGHRPV
9	1285–1362	TFELTDIVHCRMAAPSQRKAVLST-LVGRYGGRTKLYNASHSDVRDSLA-RFIPAIGPVQVTTCELYELVEAMVE-KGQDG
10	1377–1410	RITFFQKDCNKFTTGETIAHGKVGQ-GISAWSKTF
11	1421–1446	IEKAILALLPQGVFYGDAFDDTVFSA
12	1505–1518	PKESLRGFWKKHSG
ORF2		
1	25–38	PSGRRRGRRSGGSG
2	341–354	LTTTAATRFMKDLY
3	517–530	TKVTLDGRPLSTIQ
ORF3		
1	105–122	PSAPPLPHVVDLPQLGPR

was followed by four washes in water and one wash in methanol at 60°C. The blocks were then air-dried for an hour prior to storage at −20°C.

RESULTS

Pooled sera from patients with acute hepatitis E were termed "reactive" with a particular peptide only if (*i*) the optical density resulting from the interaction of the sera and peptide was ≥3-fold higher than the interaction of the same peptide with the pooled control sera and (*ii*) the peptide was adjacent to at least one other peptide that met the same criterion. Since adjacent peptides overlapped by four amino acids, significant reactivity directed against two adjacent peptides strongly suggests the presence of an epitope that is common to both peptides.

Fig. 1 depicts the optical densities at 405 nm obtained for the reactive peptides of one of the duplicate syntheses. Reactivity with antibodies is localized to epitopes contained within 12 discrete regions of the polyprotein encoded by ORF1, to three discrete regions of the presumed structural protein encoded by ORF2, and to one discrete region of the

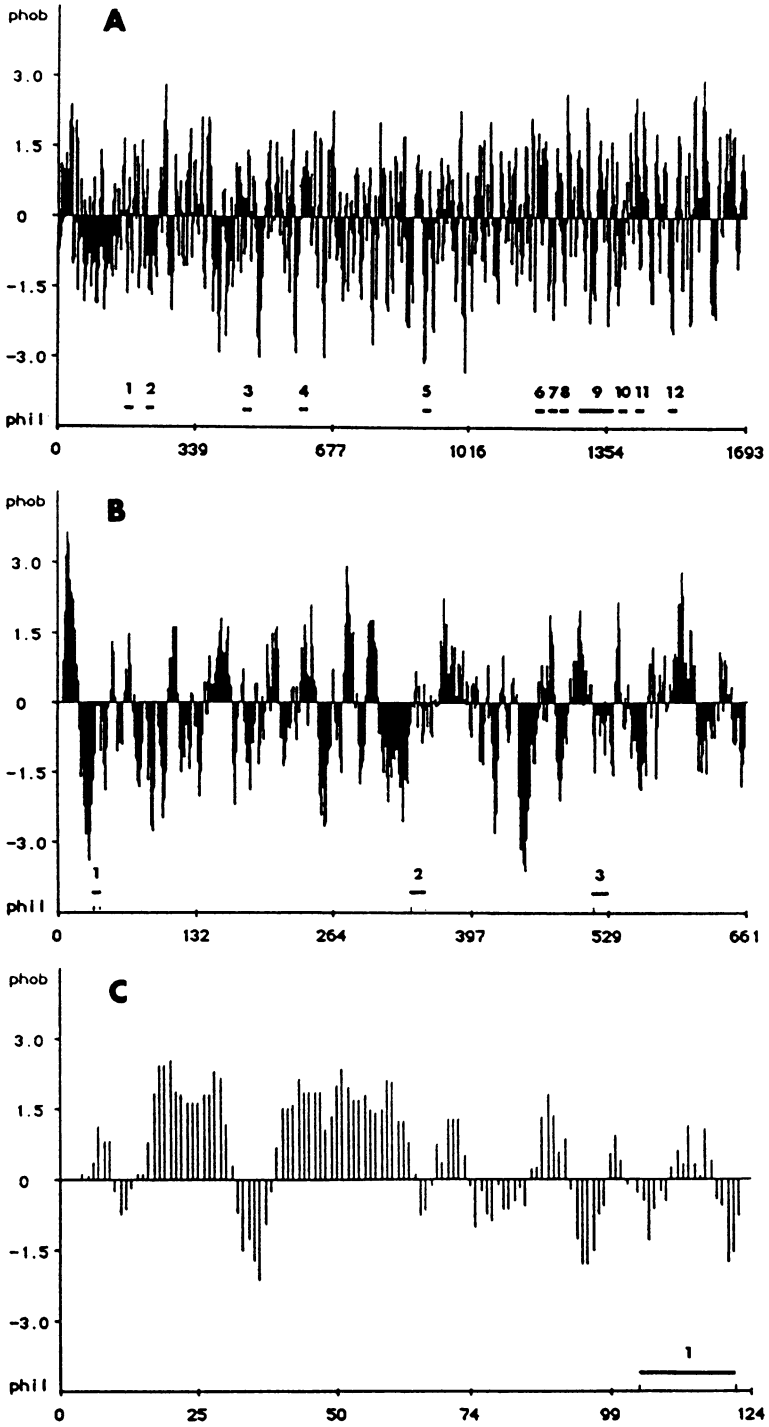

FIG. 2. Hydropathy profiles of proteins encoded by ORF1 (*A*), ORF2 (*B*), and ORF3 (*C*). Positions of the 16 regions reactive with pooled serum from patients with ET-NANBH are indicated below each profile. Positive values on the ordinate indicate hydrophobicity (phob); negative values indicate hydrophilicity (phil).

presumed structural protein encoded by ORF3. The sequence of each of these regions is listed in Table 1.

The hydropathy profiles (21) of the proteins encoded by the three ORFs are shown in Fig. 2. Analysis of each of the 16 reactive regions described in Table 1 indicates that each region contains at least one hydrophilic locus.

DISCUSSION

The present study precisely defines linear human B-cell epitopes on proteins encoded by HEV by examining the reactivities of synthetic peptides with pooled sera obtained from patients with acute infection. The presence of at least one hydrophilic site in each of the 16 reactive regions identified in the present study is consistent with our current understanding of the structure of B-cell epitopes (21). Furthermore, comparison of the sequences of each of these epitopes with a protein data base (Swiss-Prot, August 1991) suggests that these epitopes are unique to HEV with only minor homologies to previously reported proteins. A previous study examined the reactivities of sera from acutely infected patients with recombinant HEV proteins expressed in *Escherichia coli* (18, 19). The epitope on the structural protein encoded by ORF3 (amino acids 105–122) identified in the present study is located within an epitope (amino acids 91–123) identified on a protein expressed by the recombinant λgt11 clone 406.4-2(B) (18). However, a prominent epitope defined in the ORF2-encoded structural protein (amino acids 613–660) expressed by the recombinant λgt11 clone 406.3-2(B) (18) was not identified in the present study. The absence of reactivity to peptides synthesized in this region of ORF2 in the present study may be due to the lack of a proper conformation required for recognition of this epitope or, alternatively, to type-specific immune responses to geographically different strains of HEV. The present study is somewhat limited because it identifies linear epitopes only and not conformational epitopes. Since HEV cannot be propagated *in vitro* and because of the scarcity of viral particles in stool samples from infected individuals (3), it is not possible at the present time to map conformational epitopes that may be encoded by the virus.

Based on homologies of nonstructural proteins encoded by other viruses, it is hypothesized that ORF1 encodes nonstructural viral proteins, including a helicase (16, 17) and an adjacent RDRP (15–17). Although the gene encoding the RDRP is located 3′ to the helicase gene (17), it has not been precisely determined where the helicase gene ends and where the RDRP begins. The identification of consensus proteolytic sites (26) suggests that the RDRP begins approximately at amino acid 1207 of the deduced protein encoded by ORF1. This would place a very concentrated region of human B-cell epitopes (amino acids 1207–1446) entirely within the RDRP. Although an antibody response directed against an RDRP (the "virus infection-associated" or VIA antigen) has been described in a guinea pig model of infection with foot-and-mouth-disease virus (22), a prominent acute human antibody response directed against an RDRP has not been described; nor is it clear why the acute immune response should target the RDRP. In the guinea pig model of infection with foot-and-mouth-disease virus, viral replication *in vivo* was clearly required for the generation of antibodies against the VIA antigen (23). Perhaps the prominent response against the RDRP represents a nonprotective response against a highly antigenic region of the polyprotein, or perhaps antigens derived from this region are processed and appear on the surface of infected cells to serve as antigenic sites for various antibody-dependent antiviral immune mechanisms such as antibody-dependent cytotoxicity (24). Alternatively, the RDRP may be virion-associated as has been described for other viruses (25). Confirmation of these hypotheses awaits the ability to propagate HEV *in vitro*.

We thank Dr. Edward Oaks for helpful discussion, Stephanie Grays for outstanding technical support, and Dr. James Woody for support and encouragement. This investigation was supported by the Naval Medical Research and Development Command, Department of the Navy, Research Task no. 3M162787A870.AQ-208.

1. Vishwanathan, R. (1957) *Indian J. Med. Res.* **45,** Suppl., 1–30.
2. Hillis, A., Shrestha, S. M. & Saha, N. K. (1973) *J. Nepal Med. Assoc.* **11,** 145–151.
3. Kane, M. A., Bradley, D. W., Shrestha, S. M., Maynard, J. E., Cook, E. H., Mishra, R. P. & Joshi, D. D. (1984) *J. Am. Med. Assoc.* **252,** 3140–3145.
4. Balayan, M. S., Andjaparidze, A. G., Savinskaja, S. S., Ketiladze, E. S., Braginsky, D. M., Savinov, A. P. & Poleschuk, V. F. (1983) *Intervirology* **20,** 23–31.
5. Tavera, C., Velazquez, O., Avila, C., Ornelas, G., Alvarez, C. & Sepulveda, J. (1987) *Morbidity and Mortality Weekly Report* (Centers for Disease Control, Atlanta), Rep. 36, pp. 597–602.
6. Belabbes, E. H., Bouguermouh, A., Benatallah, A. & Illoul, G. (1985) *J. Med. Virol.* **16,** 257–263.
7. Bradley, D. W. & Maynard, J. E. (1986) *Semin. Liver Dis.* **6,** 56–66.
8. Bradley, D. W. (1990) *Br. Med. Bull.* **46,** 442–461.
9. Khuroo, M. S., Duermeyer, W., Zargar, S. A., Ahanger, M. A. & Shah, M. A. (1983) *Am. J. Epidemiol.* **118,** 360–364.
10. Myint, H., Soe, M. M., Khin, T., Myint, T. M. & Tin, K. M. (1985) *Am. J. Trop. Med. Hyg.* **34,** 1183–1189.
11. Purcell, R. H. & Ticehurst, J. R. (1988) *Viral Hepatitis and Liver Disease* (Liss, New York), pp. 131–137.
12. Bradley, D. W. & Balayan, M. S. (1988) *Lancet* **i,** 819.
13. Bradley, D. W., Krawczynski, K., Cook, E. H., Jr., McCaustlan, K. A., Humphrey, C. D., Spelbring, J. E., Myint, H. & Maynard, J. E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6277–6281.
14. Arankalle, V. A., Ticehurst, J., Sreenivasan, M. A., Kapikiyan, A. Z., Popper, H., Pavri, K. M. & Purcell, R. H. (1988) *Lancet* **i,** 550–554.
15. Reyes, G. R., Purdy, M. A., Kim, J. P., Luk, K.-C., Young, L. M., Fry, K. E. & Bradley, D. W. (1990) *Science* **247,** 1335–1339.
16. Fry, K. E., Tam, A. L., Smith, M. M., Kim, J. P., Luk, K.-C., Young, L. M., Piatak, M., Feldman, R. A., Yun, K. Y., Purdy, M. A., McCaustland, K., Bradley, D. W. & Reyes, G. R. (1992) *Virus Genes*, in press.
17. Tam, A. W., Smith, M. M., Guerra, M. E., Huang, C. C., Bradley, D. W., Fry, K. E. & Reyes, G. R. (1991) *Virology* **185,** 120–131.
18. Yarbough, P. O., Tam, A. W., Fry, K. E., Krawczynski, K., McCaustland, K. A., Bradley, D. W. & Reyes, G. R. (1991) *J. Virol.* **65,** in press.
19. Hyams, K. C., Purdy, M. A., Kaur, M., McCarthy, M. C., Krawczynski, K., Bradley, D. W. & Carl, M. (1992) *J. Infect. Disease*, in press.
20. Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbick, G. & Schoofs, P. G. (1987) *J. Immunol. Methods* **102,** 259–274.
21. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
22. Newman, J. F. E., Cartwright, B., Doel, T. R. & Brown, F. (1979) *J. Gen. Virol.* **45,** 497–507.
23. Polatnick, J., Arlinghaus, R. B., Graves, J. H. & Cowan, K. M. (1967) *Virology* **31,** 609–615.
24. Sissons, J. G. P. & Oldstone, M. B. A. (1980) *Adv. Immunol.* **29,** 209–260.
25. Sun, W. & Gong, Z. K. (1988) *Intervirology* **29,** 154–161.
26. Koonin, E. V. (1991) *J. Gen. Virol.* **72,** 2197–2206.