Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3091–3095, May 1983
Microbiology

Serotype-specific glycoprotein of simian 11 rotavirus: Coding assignment and gene sequence

(antigen/gene coding)

G. W. BOTH*, J. S. MATTICK*, AND A. R. BELLAMY†

*Commonwealth Scientific and Industrial Research Organisation, Molecular and Cellular Biology Unit, North Ryde, New South Wales, 2113, Australia; and †Department of Cell Biology, University of Auckland, Auckland, New Zealand

Communicated by W. K. Joklik, February 17, 1983

ABSTRACT **Cloned DNA copies of the double-stranded RNA genomic segments of simian 11 rotavirus have been used to determine the coding assignment for VP7, the type-specific antigen of this virus. Translation of hybrid-selected mRNAs in an *in vitro* system supplemented with canine pancreatic microsomes permitted VP7 to be assigned to segment 9 and the two nonstructural viral proteins NCVP4 and NCVP3, to segments 7 and 8, respectively. Hybridization of cloned DNA probes for segments 7–9 with the corresponding segments of human rotavirus Wa confirmed these assignments. The complete nucleotide sequence of gene 9 has been determined. The deduced amino acid sequence reveals VP7 to be 326 amino acids in length with two NH_2-terminal hydrophobic regions and a single glycosylation site at residues 69–71.**

Rotaviruses are important etiologic agents of infectious gastroenteritis in young children and animals (1, 2). The rotavirus genome consists of 11 segments of double-stranded RNA which, for simian 11 rotavirus (SA11), range from 660 to 3,720 base pairs in length (3). Neutralizing antibodies group the human rotaviruses into at least three major serotypes. On this basis, SA11 is indistinguishable from the third human serotype (4).

Two proteins comprise the outer shell of SA11 particles, the major one being a M_r 38,000 glycoprotein VP7 (5) which appears to elicit the neutralizing antibody response during infection (6). This glycoprotein is also of interest with respect to the mechanism of glycosylation because both the nature of the carbohydrate (7, 8) and the nonenveloped character of the virus (1) imply that VP7 is not processed by the Golgi system and that modification is effected only by the rough endoplasmic reticulum. For SA11, VP7 has not previously been assigned to a particular genomic segment although *in vitro* translation studies (5, 8) localized VP7 to one of genomic RNA segments 7, 8, and 9.

Here we describe the use of cloned SA11 genes to assign VP7 to genomic segment 9. The complete sequence of this segment has been determined and the amino acid sequence of the protein has been deduced. The nucleotide sequence predicts one major reading frame yielding a protein that includes two NH_2 terminal hydrophobic regions and a single potential glycosylation site.

MATERIALS AND METHODS

Growth and Purification of Virus and Preparation of Cell Lysates. SA11 was propagated in MA104 cells cultivated in minimal essential medium (GIBCO) and purified as described (9). To prepare [^{35}S]methionine-labeled viral proteins, confluent cultures of MA104 cells in 100-mm Petri dishes were infected at a multiplicity of 5 plaque-forming units per cell in minimal essential medium which lacked serum but contained trypsin at 10 μg/ml. Tunicamycin (10 μg/ml) was added to some cultures. At 6 hr after infection, the infected monolayers were washed with methionine-deficient culture medium and incubated for 2 hr with [^{35}S]methionine (10 μCi/ml; 1 Ci = 3.7 × 10^{10} Bq; Amersham) in minimal essential medium lacking methionine. Infected cells were then removed from the dishes by vigorous pipetting, washed in Tris-buffered saline, and lysed in 1% $NaDodSO_4$.

Synthesis and Purification of Viral mRNA. Viral mRNA was synthesized *in vitro* by using the endogenous transcriptase in viral cores (10). This yielded intact mRNA as judged by electrophoretic analysis under denaturing conditions (11). mRNA was freed of double-stranded RNA by precipitation from 2 M lithium chloride (12).

Genomic Clones and Hybridization Analysis. Procedures for the synthesis of double-stranded DNA copies of the double-stranded RNA genome segments and their cloning in pBR322 have been reported (3). Genomic double-stranded RNAs were resolved by polyacrylamide gel electrophoresis. Cross-hybridizations were carried out as described (9).

Hybrid Selection and Translation of Viral mRNAs. Hybrid selection of specific viral mRNAs was carried out by using cloned gene copies immobilized on nitrocellulose filters (13). Viral mRNA was translated in the presence of [^{35}S]methionine with a rabbit reticulocyte lysate system (Amersham N.90) supplemented with 100 μg of *Escherichia coli* tRNA per ml (14). For the *in vitro* glycosylation of translation products, microsomal membranes were prepared from canine pancreas (15). Translation products were displayed on a $NaDodSO_4$/urea/polyacrylamide gradient gel system (16).

Nucleic Acid Sequence Determination. The sequence of the cloned DNA copy of gene 9 was determined by using a combination of the methods of Maxam and Gilbert (17) and Sanger *et al.* (18) after subcloning of fragments into the phage M13mp7.1 (19). Partial sequences were determined indirectly from viral mRNA by the dideoxy method with DNA restriction fragments as primers for reverse transcription (20).

RESULTS

Coding assignments of genomic segments 7, 8, and 9

In a previous report (3) we described the synthesis, cloning, and characterization of DNA copies for SA11 genomic segments *8*, *9*, *10*, and *11*. We subsequently obtained a full-length

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: DBM, diazobenzyloxymethyl; SA11, simian 11 rotavirus.

copy of genomic segment 7. These gene copies have been used to establish precise coding assignments for SA11 genes 7, 8, and 9 by two approaches. The cloned DNAs have been used (*i*) to select specific mRNAs for analysis by *in vitro* translation and (*ii*) to enable cross-hybridization experiments to be carried out with a related rotavirus strain for which the assignment of the neutralizing activity is known (21).

Coding Assignment by Translation of Hybrid-Selected SA11 mRNAs. It has been shown previously that proteins translated *in vitro* can be glycosylated by using microsomal membranes from pancreatic endoplasmic reticulum (15). Because two of the SA11 genes 7, 8, and 9 code for nonstructural proteins which are nonglycosylated and one codes for the glycoprotein VP7 (8), only one of the protein products synthesized *in vitro* under the direction of the appropriate hybrid-selected mRNA should be modified by microsomal membranes.

Cloned DNA copies for genes 7, 8, and 9 were used to select mRNAs specific for each of these genes. DNA for each cloned gene was bound to nitrocellulose filters which were then incubated with SA11 mRNA under conditions that allowed only the homologous mRNAs to hybridize to the DNA. After stringent washing, the mRNA bound to each filter was recovered (13). These mRNAs were then translated in reticulocyte lysates in the presence or absence of microsomal membranes.

Different major products were translated in response to each of the mRNAs for genes 7, 8, and 9 (Fig. 1). However, only the gene 7 protein was clearly visible in the *in vitro* products synthesized under the direction of total SA11 mRNA. This may reflect a greater inherent translation efficiency for gene 7 and other mRNAs with the result that the translation of genes 8 and 9 is decreased. Other minor translation products present are almost certainly derived from incomplete mRNA transcripts in the mRNA pool.

Based on migration of the major *in vitro* products relative to the proteins in infected cells (Fig. 1, lane k), genes 7 and 8 code for the nonstructural viral proteins NCVP4 and NCVP3, respectively. By inference, the translation product obtained from gene 9 mRNA should represent the apoprotein of VP7. However, the *in vitro* product lacks carbohydrate and cannot be directly correlated with the mature form obtained from infected cell lysates. This uncertainty was overcome by the addition of microsomal membranes to the translation system. Only the product encoded by gene 9 was modified in the presence of microsomal membranes, indicating that this protein possesses the necessary signal for glycosylation. Furthermore, the modified gene 9 product now migrated in the gel with authentic VP7. These results confirm the assignment of VP7 to genomic segment 9.

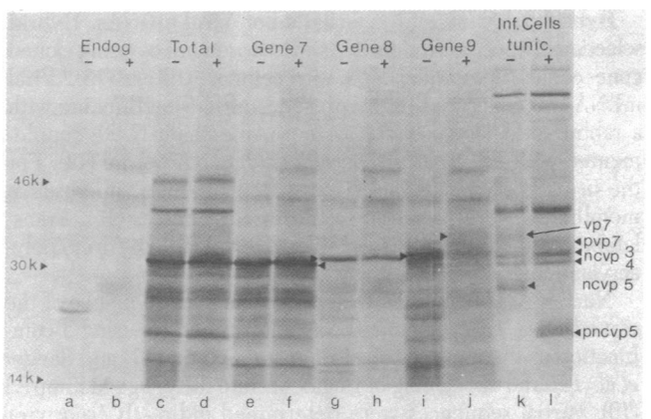

FIG. 1. Translation of hybrid-selected SA11 mRNAs. Translations were carried out in reticulocyte lysates in the presence (+) or absence (−) of canine pancreatic microsomal membranes in response to the following added mRNAs: lanes a and b, none; lanes c and d, total SA11 mRNA; lanes e–j, mRNA hybrid selected by DNA derived from cloned gene 7 (lanes e and f), gene 8 (lanes g and h), or gene 9 (lanes i and j). The viral proteins present in [^{35}S]methionine-labeled infected cells cultured in the presence (+) or absence (−) of tunicamycin are shown in lanes l and k, respectively. Arrowheads at the right indicate the position of viral proteins VP7, pVP7, NCVP3, NCVP4, NCVP5, and pNCVP5 (nomenclature of refs. 2, 5, and 8). pVP7 and pNCVP5 refer to the nonglycosylated and unprocessed forms of VP7 and NCVP5, respectively. Arrowheads at the left of the figure indicate the position of standard M_r markers (shown × 10^{-3}).

Coding Assignment by Gene Hybridization. Analysis of SA11 and human Wa rotavirus proteins by limited proteolytic digestion (22) has shown that the two nonstructural proteins encoded by genes 7, 8, and 9 are similar whereas the two VP7 proteins are markedly different. If similarities in protein sequence are conserved in the gene then the respective nonstructural genes should show nucleic acid sequence homology whereas the two VP7 genes should not. Because the neutralization specificity of the Wa strain is known, from genetic studies, to segregate with gene 9 (21), cross-hybridization of segments 7, 8, and 9 from these two viruses should enable the neutralizing gene of SA11 to be assigned to a particular segment. Equivalent amounts of genomic double-stranded RNA from SA11 and Wa rotaviruses were separated by polyacrylamide gel electrophoresis and transferred electrophoretically to diazobenzyloxymethyl (DBM) paper. The cloned DNA copies of SA11 segments 7, 8, and 9 were radiolabeled by nick-translation (23) and an equivalent amount of each gene-specific probe was hybridized individually to Wa or SA11 genome RNA segments on separate DBM-paper strips (9). Homologous and heterologous hybridizations were carried out in 50% and 10% formamide, respectively. Thus, the heterologous conditions were less stringent than the homologous hybridizations, which improved the chances for detection of sequence homology.

Cloned SA11 segments 7 and 8 hybridized specifically to the equivalent RNA segments of the Wa virus (Fig. 2). In contrast, SA11 segment 9 hybridized to its homologous RNA segment but not to any Wa RNA segment in the cluster. These results indicate that genes 7, 8, and 9 of SA11 are equivalent to the corresponding genes of the human strain Wa. Segment 9 therefore codes for the serotype-specific protein of both viruses.

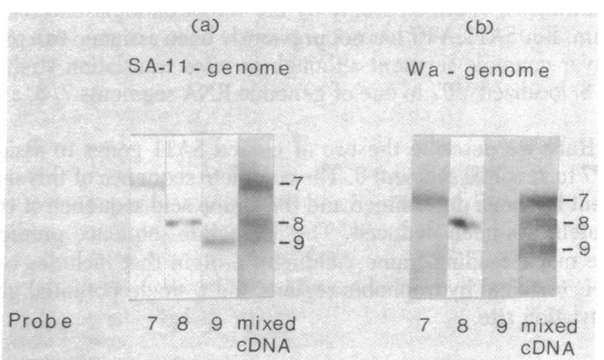

FIG. 2. Cross-hybridization of the genomes of SA11 and human Wa rotavirus. Genomic RNA of each virus was resolved by polyacrylamide gel electrophoresis and transferred to DBM-paper by transverse electrophoresis (9). ^{32}P-Labeled cloned DNA corresponding to SA11 genes 7, 8, and 9 was hybridized to individual strips by using standard procedures (3, 23). An additional DBM-paper strip of each resolved genome received a mixed homologous cDNA probe (9) to locate the positions of each gene on the paper. Homologous hybridizations (*a*) were carried out in 50% formamide at 52°C; heterologous hybridizations (*b*) were in 10% formamide at 52°C.

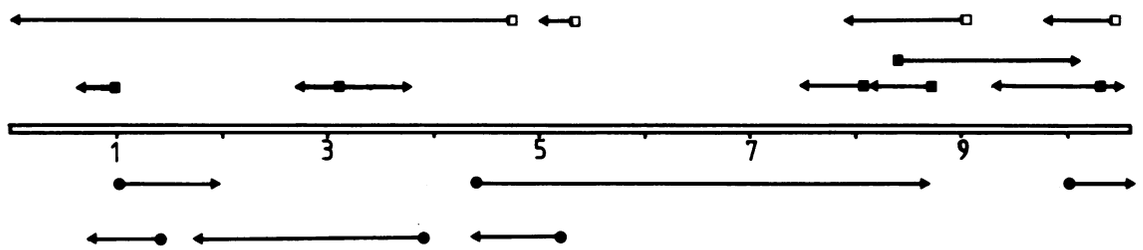

FIG. 3. Strategy for determining the nucleotide sequence of the cloned DNA copy of double-stranded RNA gene segment *9* of SA11. The numbers refer to the distance in nucleotides × 10^{-2}. The sequence was determined by copying cDNA from SA11 mRNA (□) (20), by the Maxam and Gilbert methods (17) (■), or by the dideoxy method (18) after subcloning into phage m13mp7.1 (19) (●).

```
                                                                M  Y  G  I  E  Y  T  T  V  L  T  F  L  I
GGCUUUAAAAGAGAGAAUUUCCGUUUGGCUAGCGGUUAGCUCCUUUUAAUGUAUGGUAUUGAAUAUACCACAGUUCUAACCUUUCUGAUA
                    20                  40                  60                  80

 S  I  I  L  L  N  Y  I  L  K  S  L  T  R  I  M  D  C  I  I  Y  R  L  L  F  I  I  V  I  L
UCGAUUAUUCUACUAAAUUACAUACUUAAAUCAUUAACUAGAAUAAUGGACUGUAUAAUUUAUAGAUUGCUUUUUAUAAUUGUGAUAUUG
        100                 120                 140                 160                 180

 S  P  F  L  R  A  Q  N  Y  G  I  N  L  P  I  T  G  S  M  D  T  A  Y  A  N  S  T  Q  E  E
UCACCAUUUCUCAGAGCACAAAAUUAUGGUAUUAAUCUUCCAAUCACAGGCUCCAUGGACACUGCAUACGCUAAUUCAACGCAAGAAGAA
                200                 220                 240                 260

 T  F  L  T  S  T  L  C  L  Y  Y  P  T  E  A  A  T  E  I  N  D  N  S  W  K  D  T  L  S  Q
ACAUUCCUCACUUCUACACUUUGCCUAUAUUAUCCGACUGAGGCUGCGACUGAAAUAAACGAUAAUUCAUGGAAAGACACACUGUCACAA
        280                 300                 320                 340                 360

 L  F  L  T  K  G  W  P  T  G  S  V  Y  F  K  E  Y  T  N  I  A  S  F  S  V  D  P  Q  L  Y
CUAUUUCUUACGAAAGGGUGGCCAACUGGAUCCGUAUAUUUUAAAGAAUAUACUAACAUUGCAUCGUUUUCUGUUGAUCCGCAGUUGUAU
                380                 400                 420                 440

 C  D  Y  N  V  V  L  M  K  Y  D  A  T  L  Q  L  D  M  S  E  L  A  D  L  I  L  N  E  W  L
UGUGAUUAUAACGUAGUACUAAUGAAAUAUGACGCGACGUUGCAAUUGGAUAUGUCAGAACUUGCGGAUCUAAUAUUAAACGAAUGGUUG
        460                 480                 500                 520                 540

 C  N  P  M  D  I  T  L  Y  Y  Y  Q  Q  T  D  E  A  N  K  W  I  S  M  G  S  S  C  T  I  K
UGUAAUCCAAUGGAUAUUACUCUGUAUUAUUAUCAGCAAACUGACGAAGCGAAUAAAUGGAUAUCAAUGGGCUCAUCAUGUACAAUUAAA
                560                 580                 600                 620

 V  C  P  L  N  T  Q  T  L  G  I  G  C  L  T  T  D  A  T  T  F  E  E  V  A  T  A  E  K  L
GUAUGUCCACUUAAUACACAAACUCUUGGAAUUGGAUGCUUGACAACUGAUGCUACAACUUUUGAAGAAGUUGCGACAGCUGAAAAGUUG
        640                 660                 680                 700                 720

 V  I  T  D  V  V  D  G  V  N  H  K  L  D  V  T  T  A  T  C  T  I  R  N  C  K  K  L  G  P
GUAAUUACUGACGUGGUUGAUGGCGUUAAUCAUAAGCUGGAUGUCACAACAGCAACGUGUACUAUUAGAAACUGUAAGAAAUUGGGACCA
                740                 760                 780                 800

 R  E  N  V  A  V  I  Q  V  G  G  S  D  I  L  D  I  T  A  D  P  T  T  A  P  Q  T  E  R  M
AGAGAAAACGUAGCCGUUAUACAAGUUGGUGGUUCUGACAUCCUCGAUAUAACUGCUGAUCCAACUACUGCACCACAGACAGAACGGAUG
        820                 840                 860                 880                 900

 M  R  I  N  W  K  K  W  W  Q  V  F  Y  T  V  V  D  Y  V  D  Q  I  I  Q  V  M  S  K  R  S
AUGCGAAUUAACUGGAAAAAAUGGUGGCAAGUUUUUUAUACUGUAGUAGACUAUGUAGAUCAGAUAAUACAAGUUAUGUCCAAAAGAUCA
                920                 940                 960                 980

 R  S  L  N  S  A  A  F  Y  Y  R  V  *
AGAUCACUAAAUUCAGCAGCAUUUUAUUACAGAGUGUAGGUAUAACUUAGGUUAGAAUUGUAUGAUGUGACC
      1000                1020                1040                1060
```

FIG. 4. Nucleotide sequence of the (+) (coding) strand of SA11 RNA gene *9* and the predicted amino acid sequence of the VP7 protein. Overbars identify blocks of amino acids in regions of high hydrophobicity at the NH_2 terminus of VP7 as determined by a computer-generated Kyte–Doolittle plot (7, 24). The broken line indicates the glycosylation site.

Structure and sequence of SA11 gene 9

Preliminary characterization of the cloned DNA copy of gene 9 showed that it represented all but 70 base pairs of the double-stranded RNA segment (3). The sequence of the gene was determined by the strategy outlined in Fig. 3. For the most part the sequence was determined either on both strands of the cloned DNA or by more than one method, except for a small region between bases 520 and 720 where the M13 sequence data were clear and unambiguous. The sequence of the nucleotides missing from the clone was determined indirectly from SA11 mRNA. This was achieved by first preparing from cloned gene 9 a *Dde* I–*Taq* I fragment complementary to bases 90–190 of the gene 9 (+) strand. This primer was then hybridized to total SA11 mRNA and elongated by reverse transcriptase in the presence of dideoxy nucleoside triphosphates to generate the missing sequence data (18, 20). The sequence complementary to the first three nucleotides (G-G-C) of the mRNA could not be determined by this method and has been inferred from the presence of this conserved 5′ terminal sequence on all other cloned SA11 genes so far examined (3). The complete nucleotide sequence for the (+) strand of gene 9 and the protein sequence it predicts are shown in Fig. 4. The RNA segment is 1,062 bases long and has 5′ and 3′ noncoding regions of 48 and 36 bases. There is one major open reading frame which codes for a polypeptide of 326 amino acids. The other two reading frames are open for no more than 50 codons.

DISCUSSION

The coding assignment for the type-specific glycoprotein VP7 has been obtained by using two independent approaches which both assign this viral protein to genomic segment 9. In addition, genes 7 and 8 have been found to code for the two nonstructural viral proteins NCVP4 and NCVP3, respectively. The reason for the diffuse nature of the protein product translated from gene 9 mRNA (Fig. 1) is not clear, nor is it evident why this product should be slightly smaller than the VP7 precursor protein synthesized in SA11-infected cells in the presence of tunicamycin (Fig. 2) (5, 8). Nevertheless, VP7 is the only protein product of this group of genes which is modified by the addition of microsomal membranes, and the glycosylated product is similar in size to authentic VP7. Taken together with the hybridization result, *in vitro* translation therefore assigns VP7 unequivocally to gene 9.

There are several features of the gene 9 nucleotide sequence and the inferred protein sequence that merit comment. First, flanking nucleotides near the first AUG in the open reading frame do not conform to the consensus sequence for eukaryotic initiation codons (25) but those near the second AUG at bases 136–138 do fit this pattern. Although we do not yet know which initiation codon is used, the relative M_r estimates for pVP7 (35,500), NCVP3 (35,000), and NCVP4 (34,000) (5, 8) are consistent with the estimates based on the longest open reading frame for genes 9, 8, and 7, respectively (ref. 3; unpublished data), suggesting that the first initiation codon is used in gene 9. Second, the two regions, approximately 18 amino acids long, that follow each methionine residue are hydrophobic and both therefore have the superficial features of the signal sequence required for the transport of a protein through a membrane (26). The presence of a putative signal peptide at the NH_2 terminus is analogous to the situation that exists for other viral proteins such as the hemagglutinin of influenza virus (27) and the glycoproteins of vesicular stomatitis virus (28) and Semliki Forest virus (29). Further work is required to establish which potential initiation codons are used in the translation of VP7 and how much processing of the NH_2 terminus of this glycoprotein occurs during virus maturation.

In contrast to viral glycoproteins such as those of Semliki Forest virus which appear to be anchored in the viral membrane by a sequence of hydrophobic residues near the COOH terminus of the protein (29), there is no evidence for a hydrophobic sequence at the COOH terminus of VP7. This observation might be anticipated for a rotavirus such as SA11 because, in common with other members of the family *Reoviridae*, it is nonenveloped and does not bud from the plasma membrane (1, 2).

It will be most interesting to compare the sequence of the SA11 type-specific antigen with the equivalent VP7 proteins from other rotaviruses which belong to different serotypic groups. The lack of detectable nucleotide homology between the SA11 and Wa genes observed in this study suggests that extensive sequence divergence will be found between different rotavirus serotypic proteins. The availability of sequence information for the immunologically important genes of this clinically significant group of viruses should also enable the eventual production of proteins or peptides that could be of potential value for vaccine purposes.

We thank Jeanette Street, Linda Siegman, William Chadderton, and Elizabeth Hamilton for skilled and enthusiastic technical assistance and Drs. P. Molloy, D. Lane, and R. E. F. Matthews for comments on the manuscript. This work was supported by grants from the New Zealand Medical Research Council, National Child Health Research Foundation of New Zealand, and World Health Organization.

1. Holmes, I. H. (1979) *Prog. Med. Virol.* **25**, 1–36.
2. Estes, M. K., Palmer, B. L. & Obijeski, J. F. (1982) *Curr. Top. Microbiol. Immunol.*, in press.
3. Both, G. W., Bellamy, A. R., Street, J. E. & Siegman, L. J. (1982) *Nucleic Acids Res.* **10**, 7075–7088.
4. Wyatt, R. G., Greenberg, H. B., James, W. D., Pittman, A. L., Kalica, A. R., Flores, J., Chanock, R. M. & Kapikian, A. Z. (1982) *Infect. Immun.* **37**, 110–115.
5. Ericson, B. L., Graham, D. Y., Mason, B. B. & Estes, M. K. (1982) *J. Virol.* **42**, 825–839.
6. Bastardo, J. W., McKinn-Breschkin, J. L., Sonza, S., Mercer, L. D. & Holmes, I. H. (1981) *Infect. Immun.* **34**, 641–647.
7. Both, G. W., Mattick, J. S., Siegman, L. J., Atkinson, P. H., Weiss, S., Bellamy, A. R., Street, J. E. & Metcalf, P. (1982) in *The Double-Stranded RNA Viruses*, eds. Bishop, D. H. L. & Compans, R. W. (Academic, New York), in press.
8. Arias, C. F., Lopez, S. & Espejo, R. T. (1982) *J. Virol.* **41**, 42–50.
9. Street, J. E., Croxson, M. C., Chadderton, W. F. & Bellamy, A. R. (1982) *J. Virol.* **43**, 369–385.
10. Mason, B. B., Graham, D. Y. & Estes, M. K. (1980) *J. Virol.* **33**, 1111–1121.
11. Sanger, F. & Coulson, A. R. (1978) *FEBS Lett.* **87**, 107–110.
12. Baltimore, D. (1966) *J. Mol. Biol.* **18**, 421–428.
13. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) *Cell* **20**, 95–105.
14. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
15. Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G. & Lodish, H. F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3278–3282.
16. Mattick, J. S., Zehner, Z. E., Calabro, M. A. & Wakil, S. J. (1981) *Eur. J. Biochem.* **114**, 643–651.
17. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Messing, J., Crea, R. & Seeburg, P. H. (1981) *Nucleic Acids Res.* **9**, 309–321.
20. Sleigh, M. J., Both, G. W., Underwood, P. A. & Bender, V. J. (1981) *J. Virol.* **37**, 845–853.
21. Kalica, A. R., Greenberg, H. B., Wyatt, R. G., Flores, J., Sereno, M. M., Kapikian, A. Z. & Chanock, R. M. (1981) *Virology* **112**, 385–390.

22. Dyall-Smith, M. L. & Holmes, I. H. (1981) *J. Virol.* **40,** 720–728.
23. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113,** 237–251.
24. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
25. Kozak, M. (1981) *Nucleic Acids Res.* **9,** 5233–5252.
26. Kreil, G. (1981) *Annu. Rev. Biochem.* **50,** 317–348.
27. Webster, R. G., Laver, W. G., Air, G. M. & Schild, G. C. (1982) *Nature (London)* **296,** 115–121.
28. Rose, J. K., Welch, W. J., Sefton, B. M., Esch, F. S. & Ling, N. C. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3884–3888.
29. Garoff, H., Frischauf, A.-M., Simons, K., Lehrach, H. & Delius, H. (1980) *Nature (London)* **288,** 236–241.