The EMBO Journal vol.13 no.4 pp.924–927, 1994

Role for poliovirus protease 2A in cap independent translation

A.J.Macadam[1], G.Ferguson, T.Fleming, D.M.Stone[2], J.W.Almond[2] and P.D.Minor

National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts EN6 3QG and [2]Department of Microbiology, University of Reading, Whiteknights, PO Box 228, Reading RG6 5AJ, UK

[1]Corresponding author

Communicated by R.J.Jackson

Viral protein synthesis in poliovirus infected cells was found to be influenced by mutations in part of the viral 5′-non-coding region (NCR) in a temperature dependent manner. At elevated temperatures these mutations resulted in virus titre reductions that allowed selection of revertant viruses. Some revertants were found to have retained the 5′-NCR mutations but had compensating mutations in the 2A protease gene that were responsible for the suppression of the temperature sensitive phenotypes. The mutations in 2A enhanced viral protein synthesis at a stage when cap dependent translation was already abolished, suggesting that the virally encoded protein 2A is directly involved in the process of cap independent translation in addition to its role in abolishing cap dependent translation.

Key words: cap independent/non-coding/poliovirus/protease/translation

Introduction

Unlike many other eukaryotic mRNAs the genomic RNA of poliovirus is translated by a cap independent mechanism in which the 5′-non-coding region functions as an internal ribosomal entry site (Pelletier and Sonenberg, 1988). Moreover, early in infection cap dependent translation in the host cells is abolished by a process involving the virally encoded protease 2A (Bernstein *et al.*, 1985) enabling the viral RNA to be efficiently translated in the presence of an excess of endogenous capped mRNAs. The mechanisms by which cap dependent translation is abolished in an infected cell and poliovirus RNA is translated in a cap independent manner are only partially understood and are likely to have significance for translation in uninfected cells in which, under certain conditions, cap dependent translation is shut down (Bonneau and Sonenberg, 1987; Lamphear and Panniers, 1991). In these circumstances cap independent translation of some cellular mRNAs is likely and has been demonstrated in at least one case (Macejak and Sarnow, 1991).

Cap independent translation of poliovirus RNA is governed in part by the ~740 base 5′-non-coding region (NCR) which is known to have extensive secondary structure (Skinner *et al.*, 1989). There is evidence that disruption of this structure can lead to reduced translational efficiency (La Monica and Racaniello, 1989; Svitkin *et al.*, 1990; Percy *et al.*, 1992) and significant biological effects, including important contributions to the avirulent phenotypes of the highly successful live oral vaccine strains of poliovirus (Minor, 1992). It has previously been shown that mutations which weaken the secondary structure of the 5′-NCR of poliovirus can produce viruses whose growth is sensitive to elevated temperatures in certain cell types (Macadam *et al.*, 1991a, 1992). Most mutations were introduced into domain VI (nucleotides 471–538; Figure 1) which is the location of mutations known to attenuate the Sabin vaccine strains (Minor, 1992). Revertants selected *in vivo* and *in vitro* have been found to have either mutated back to wild-type sequence or introduced compensating mutations that restored base pairing (Minor and Dunn, 1988; Skinner *et al.*, 1989; Macadam *et al.*, 1992). A third category of revertant is described here.

Results

Selection of revertants

The parental viruses from which revertants were selected were either serotype 2 viruses deriving the 5′-491 nucleotides of the genome from the Sabin type 2 vaccine strain, P2/Sabin, or they were site-directed mutants of Leon/Lansing, a recombinant virus deriving its 5′-NCR (~750 bases) from the type 3 strain P3/Leon and the remainder of its genome from the type 2 strain Lansing (Table I; Figure 1). Mutant LL472/537UG had a U at 472 as in the type 3 vaccine strain rather than a C as in the wild-type; mutants LLΔ472 and LLΔ483 had single base deletions at 472 and 483, respectively; mutant LL479/532UC had substitutions at both 479 and 532, creating a mismatched UC base pair and LL514A had a single mutation U→A at 514 (Figure 1). As a result of these 5′-NCR sequences growth in BGM cells of all parental viruses was sensitive to elevated temperatures to some degree, as illustrated by the ratios of numbers of plaques formed at 35°C and 39°C (Table I). In contrast, growth of the strains in which the predicted structure of the 5′-NCR was not disrupted such as P2/117, PS/1175′ and Leon/Lansing was similar at 39°C and 35°C.

Variants of the temperature sensitive (ts) viruses were picked following plaque-formation at 39°C or 39.3°C on BGM cells and their phenotypes are shown in Table I. It can be seen that all were markedly less ts in their growth than the viruses from which they derived. All viruses grew to similar titres and with similar plaque morphology at 35°C, except for parental viruses with deletions which had smaller plaques at this temperature.

Genetic basis of reversion

The sequences of the entire 5′-NCRs of the genomes of S2/39A, S5′/39A and S5′/39B were determined and found to be identical to the parental viruses. The sequence of the region of the genome encompassing domain VI (Figure 1) was determined for the other revertant viruses shown in

© Oxford University Press

Table I. Ts phenotypes of parent, revertant and *in vitro* reconstructed strains

Parent (precursor)	Log_{10} (p.f.u. at 35°C/p.f.u. at 39°C)	Revertant strain	Log_{10} (p.f.u. at 35°C/p.f.u. at 39°C)	*In vitro* reconstruction	Log_{10} (p.f.u. at 35°C/p.f.u. at 39°C)
(P2/117)	0.2	–	–	–	–
(PS/1175′)	0.3	–	–	S2/2A-1/1175′	0.2
P2/Sabin	2.2	S2/39A	0.6	S2/2A-1	0.5
P117/S5′	2.3	S5′/39A	0.4	S2/2A-2	0.3
		S5′/39B	0.1	S2/2A-3	0.1
		S5′/39C	0.1	–	–
		S5′/39D	0.2	–	–
(Leon/Lansing)	0.1	–	–	–	–
LL472/537UG	2.0	UG/39A	0.3	–	–
LLΔ472	4.0	Δ472/39A	0.5	–	–
LL479/537UC	2.8	UC/39A	0.2	–	–
LLΔ483	4.0	Δ483/39A	0.9	–	–
LL514A	3.0	514/39A	0.4	–	–

Table I and also shown to be identical to that of the parent from which they derived. Further sequencing of the P1 and P2 genes of S2/39A revealed a mutation in the region of the genome encoding 2A and subsequently mutations were found in the 2A genes of all revertant viruses studied here, as shown in Table II.

The 2A-encoding regions of viruses S2/39A, S5′/39A and S5′/39B were amplified by PCR and inserted into the infectious cDNA clone of P2/Sabin (Pollard *et al.*, 1989) and viruses were recovered from infectious RNA transcripts (Van der Werf *et al.*, 1986). As shown in Table I, the recovered viruses with reconstructed genotypes (S2/2A-1, -2 and -3) were identical in phenotype to the revertant viruses from which they derived their 2A genes, thus demonstrating that the mutations in 2A were responsible for the suppression of the ts phenotype attributable to the mutations in the 5′-NCR.

The effect of a 2A mutation on a virus with a wild-type 5′-NCR was examined by replacing the 2A-encoding region of PS/1175′ with that of S2/2A-1. The resultant virus (S2/2A-1/1175′) had a similar plaque morphology to PS/1175′ and S2/2A-1 at 35°C and grew to similar titres. S2/2A-1/1175′ grew as well at 39°C as 35°C like PS/1175′ and S2/2A-1 (Table I), though plaques were larger than those of PS/1175′ and S2/2A-1 at 39°C. Thus, the presence of a ts suppressor was at least not deleterious to the growth of a virus with a wild-type 5′-NCR and probably enhanced viral growth at elevated temperatures.

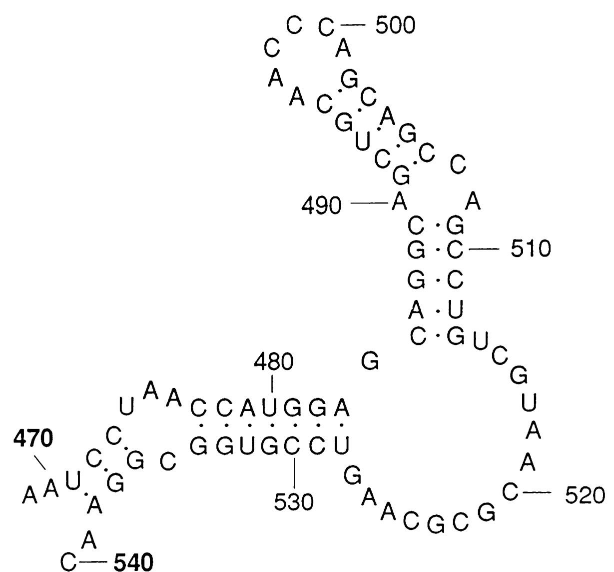

Fig. 1. Predicted secondary structure of domain VI of the poliovirus 5′-NCR (Skinner *et al.*, 1989). The sequence and numbering shown is that of P3/Leon. Nucleotide 481 in P2/Sabin is equivalent to nucleotide 484 in this figure.

Table II. Sequence differences in the 2A coding regions of revertant viruses

Isolate	Amino acid difference[a]
S2/39A	96 His-Tyr
S5′/39A	19 Tyr-His
S5′/39B	8 Ala-Val
S5′/39C	79 Thr-Ala
S5′/39D	122 Ile-Val
UG/39A	25 Glu-Gly
Δ472/39A	80 Phe-Leu
514/39A	10 Tyr-Cys
UC/39A	82 Tyr-His
Δ483/39A	23 Thr-Ile

[a]Mutations were identified by sequencing of genomic RNA through the entire 2A gene.

Protein synthesis in infected cells

The influence of 5′-NCR and 2A mutations on protein synthesis was examined by pulse-labelling of infected cells. BGM cells were infected with the type 2 Sabin strain and with the recombinant virus PS/1175′ and the revertant S2/39A whose genomes differed from P2/Sabin only in the 5′ 491 bases and 2A, respectively. After adsorption of virus for 1 h at 35°C cells were incubated at 38.5°C then pulsed with [^{35}S]methionine at 2, 2.5, 3, 3.5, 4 and 5 h post-infection. Samples of lysates from equal cell numbers were analysed by SDS–PAGE and the resulting autoradiogram is shown in Figure 2.

Synthesis of host cell proteins was shut off to a comparable degree by 2 h post-infection in all infected cells (but not at all in uninfected controls; data not shown). However, the intensity of labelling of viral proteins in the cells infected with the P2/Sabin strain was greatly reduced relative to the cells infected with either of the other two strains. Similar

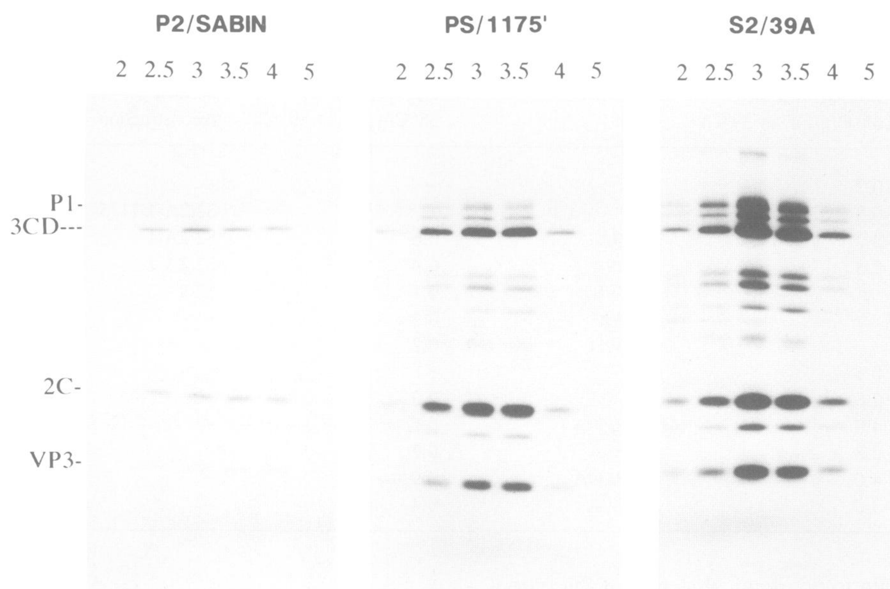

Fig. 2. Protein synthesis at 38.5°C in BGM cells infected with P2/Sabin and derivatives. These differ in sequence from P2/Sabin only in the 5′ 491 nucleotides (PS/1175′) and in 2A (S2/39A). Infected cells were pulsed with [^{35}S]methionine for 30 min at 2, 2.5, 3, 3.5, 4 and 5 h post-infection. Positions of viral polypeptides are indicated.

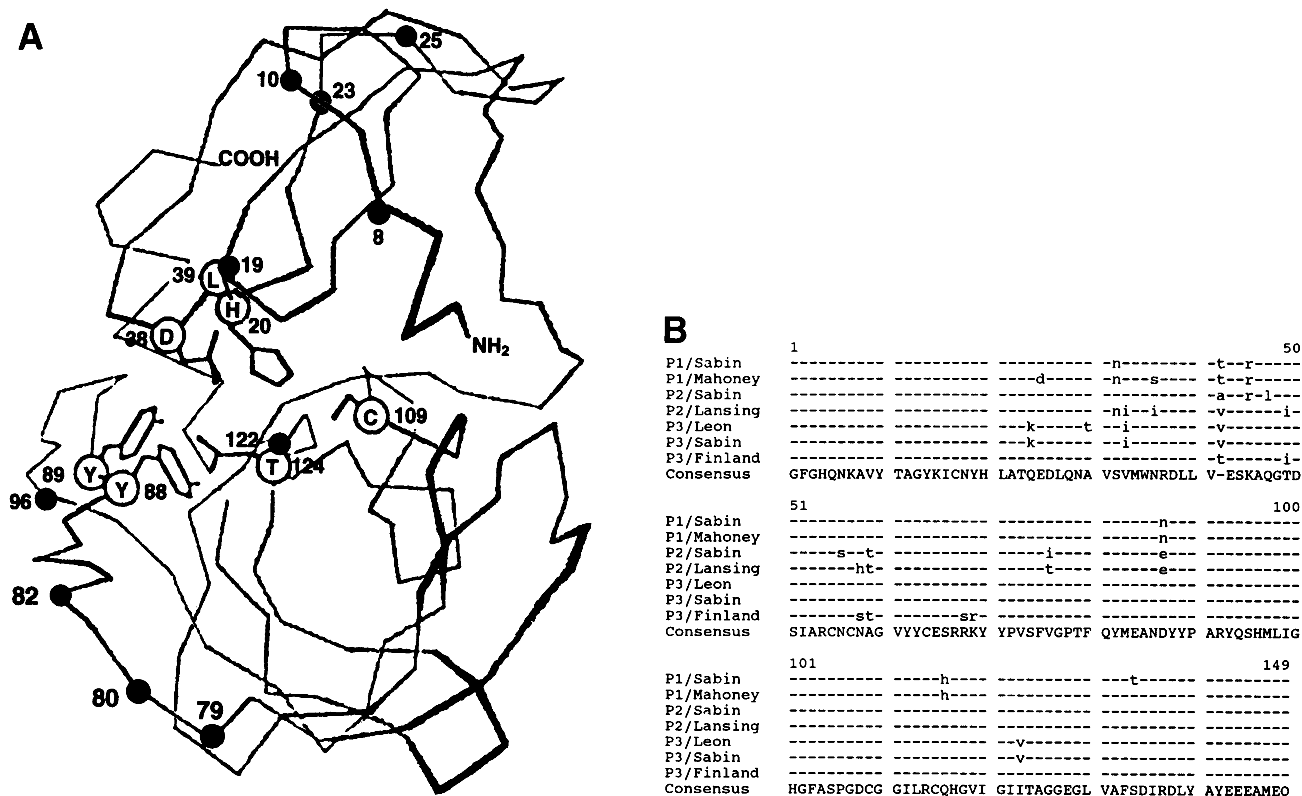

Fig. 3. Primary and secondary structure of poliovirus protease 2A. (**A**) Model of the α-carbon chain of poliovirus protease 2A based on the structure of small bacterial serine proteases (Bazan and Fletterick, 1988; Yu and Lloyd, 1991). The putative catalytic triad (residues 20, 38 and 109) and other conserved residues (39, 88, 89 and 124) are shown as well as positions of suppressor mutations (●). (**B**) Amino acid sequences of poliovirus 2A genes. Nucleotide sequences were obtained from the GenBank/EMBL database and analysed using University of Wisconsin GCG programs Translate, Lineup and Pretty. The consensus sequence is shown in upper case letters, differences in lower case and conserved residues as dashes (-).

results were obtained with other revertant strains, and also when the shift to the non-permissive temperature was performed 10 min prior to labelling (our unpublished results). This suggested that the reduction in protein synthesis in cells infected with P2/Sabin (and the other ts viruses) was due to a ts defect in translation, not RNA synthesis, since it was not reversed by allowing replication to proceed at the permissive temperature until just before [^{35}S]methionine incorporation. Thus, the 2A mutations present in S2/39A and other revertant viruses apparently suppressed the ts defect by enhancing translational efficiency at elevated temperatures.

Discussion

Results presented here and elsewhere (Macadam *et al.*, 1991a, 1992) showed that protein synthesis directed by poliovirus RNAs in intact cells is influenced by at least four interacting variables: 5'-non-coding region sequence, protease 2A sequence, temperature and host cell type. Mutations in the 5'-NCR acted through their effect on RNA secondary structure. The ability of mutations in the 2A gene to compensate for 5'-NCR destabilization is an unexpected finding whose molecular basis is not clear.

The structure of protease 2A is unknown but a model has been proposed based on the structure of small bacterial serine proteases (Bazan and Fletterick, 1988; Yu and Lloyd, 1991). Locations of 2A mutations in this model are shown in Figure 3A. In the primary sequence, and more strikingly in the structural model, most of the observed substitutions cluster in two main groups comprising residues 8, 10, 19, 23 and 25 and residues 79, 80, 82 and 96. Whereas these clusters appear to be at or near the surface of the protein the other mutation observed, at residue 122, is internal. Only residues 19 and 122 are close to the putative active site in the model.

There is only limited heterogeneity among known poliovirus sequences in the 2A region (<7% at the amino acid level), a large part of which is found between residues 30 and 50 (Figure 3B), i.e. between the two clusters of mutations observed here, so it is curious that as many as 10 different mutations were selected, all at positions conserved in other poliovirus strains. Further analysis may indicate the range of 2A substitutions involved in enhancement of protein synthesis and whether there is any specificity between 5'-NCR domain VI mutations and 2A amino acid substitutions.

Enhanced translation from a poliovirus 5'-NCR due to the presence of 2A was observed in cells still carrying out cap dependent translation (Hambridge and Sarnow, 1992). While enhancement was not due to reduced competition for the translation machinery, it was not clear whether it constituted a process separate from the mechanism of shut off itself. Indeed, it was suggested that the cleavage products of the cap binding complex might have been responsible for the transactivation by 2A. The mutations in 2A reported here enhanced viral protein synthesis at a stage when cap dependent translation was already abolished, suggesting that 2A has a direct role in cap independent translation of poliovirus RNA as well as in the shut off of cap dependent translation. While no significant homology between 2A and the common RNA recognition motif (Query *et al.*, 1989) has been found, the apparent locations of the 2A substitutions observed at or near the surface and away from the active site suggest direct interaction between 2A and the 5'-non-coding region of poliovirus in this mechanism of translation.

Materials and methods

Construction of recombinants and site-specific mutants

Construction of infectious clones of P2/Sabin and P2/117 (an isolate from a vaccine-associated case of poliomyelitis) was described by Pollard *et al.* (1989). PS/1175' and P117/S5' are reciprocal recombinants in which the first 491 nucleotides were exchanged (Macadam *et al.*, 1991a). In this region the two viruses differ only at positions 437 and 481. Leon/Lansing is a chimeric virus containing the 5'-NCR of P3/Leon and the coding and 3'-non-coding regions of P2/Lansing (equivalent to pT7SFP; Skinner *et al.*, 1989). Site-directed mutants LL472/537UG, LLΔ472 and LL479/532UC were constructed and recovered as described by Macadam *et al.* (1992). Mutants LLΔ483 and LL514A were generated by the same methods. Positions of mutations and deletions refer to nucleotide positions in P3/Leon sequence (Figure 1).

Clones S2/2A-1, -2 and -3 were constructed by replacing the *Bst*EII and *Sna*BI region (2892–4455) of the P2/Sabin clone with *Bst*EII and *Sna*BI-digested PCR fragments spanning 2A generated from revertant viral RNA. S2/2A-1/1175' was made by replacing the *Bst*EII–*Sna*BI region of PS/1175' with the equivalent region of S2/2A-1. PCR-derived regions of clones were sequenced in full and found to be identical to the parental virus (P2/Sabin or P117/S5'), except at the previously identified position in the 2A gene. Viruses were recovered by transfection of T7 transcripts (Van der Werf *et al.*, 1986) and genomic RNA was sequenced to verify the mutant genotypes.

Virus assays and mutant selection

Temperature sensitive phenotypes were determined by plaque-assay at 35°C and 39°C in BGM cells (Macadam *et al.*, 1991a). Revertants were isolated by selection of plaques in BGM cells at 39°C (UG/39A, Δ472/39A, UC/39A, Δ483/39A and 514/39A) or 39.3°C (S2/39A and S5'/39A-D).

Pulse-labelling

Monolayers of BGM cells in 25 cm^2 flasks (80–90% confluent) were infected at 35°C with viruses at a multiplicity of infection (m.o.i.) of 10 for 1 h. After addition of 5 ml prewarmed medium [Eagle's minimal essential medium (MEM) containing 1% fetal calf serum] flasks were shifted to 38.5°C by sinking in a water bath. Half an hour prior to addition of [^{35}S]methionine (Amersham, 50 μCi/flask) medium was removed and replaced by prewarmed methionine-free MEM. Labelling was at 2, 2.5, 3, 3.5, 4 and 5 h post-infection for 30 min, after which flasks were harvested by removal of medium and rapid freezing in CO_2/ethanol. Cells were lysed in 0.5 ml lysis buffer (Macadam *et al.*, 1991b) on ice and, after removal of cell debris, 50 μl aliquots were analysed by SDS–PAGE.

References

Bazan,J.F. and Fletterick,R.J. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 7872–7876.

Bernstein,H.D., Sonenberg,N. and Baltimore,D. (1985) *Mol. Cell Biol.*, **5**, 2913–2923.

Bonneau,A.M. and Sonenberg,N. (1987) *J. Biol. Chem.*, **262**, 11134–11139.

Hambridge,S.J. and Sarnow,P. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 10272–10276.

La Monica,N. and Racaniello,V.R. (1989) *J. Virol.*, **63**, 2357–2360.

Lamphear,B.J. and Parmiers,R. (1991) *J. Biol. Chem.*, **266**, 2789–2794.

Macadam,A.J., Pollard,S.R., Ferguson,G., Dunn,G., Skuce,R., Almond,J.W. and Minor,P.D. (1991a) *Virology*, **181**, 451–458.

Macadam,A.J., Ferguson,G., Arnold,C. and Minor,P.D. (1991b) *J. Virol.*, **65**, 5225–5231.

Macadam,A.J., Ferguson,G., Burlison,J., Stone,D., Skuce,R., Almond,J.W. and Minor,P.D. (1992) *Virology*, **189**, 415–422.

Macejak,D.G. and Sarnow,P. (1991) *Nature*, **353**, 90–94.

Minor,P.D. (1992) *J. Gen. Virol.*, **73**, 3065–3077.

Minor,P.D. and Dunn,G. (1988) *J. Gen. Virol.*, **69**, 1091–1096.

Pelletier,J. and Sonenberg,N. (1988) *Nature*, **334**, 320–325.

Percy,N., Belsham,G.J., Brangwyn,J.K., Sullivan,M., Stone,D. and Almond,J.W. (1992) *J. Virol.*, **66**, 1695–1701.

Pollard,S.R., Dunn,G., Cammack,N., Minor,P.D. and Almond,J.W. (1989) *J. Virol.*, **63**, 4949–4951.

Query,C.C., Bentley,R.C. and Keene,J.D. (1989) *Cell*, **57**, 89–101.

Skinner,M.A., Racaniello,V.R., Dunn,G., Cooper,J., Minor,P.D. and Almond,J.W. (1989) *J. Mol. Biol.*, **207**, 379–392.

Svitkin,Y.V., Cammack,N., Minor,P.D. and Almond,J.W. (1990) *Virology*, **175**, 103–109.

Van der Werf,S., Bradley,J., Wimmer,E., Studier,F.W. and Dunn,J.J. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 2330–2334.

Yu,S.F. and Lloyd,R.E. (1991) *Virology*, **182**, 615–625.

Received on October 18, 1993