

Identification of a new element for RNA replication within the internal ribosome entry site of poliovirus RNA

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Several mutants of the Mahoney strain of poliovirus type 1 have been generated by introducing mutations into the stem-loop II (SLII) structure within the internal ribosomal entry site (IRES). Four of these mutants (SLII-1, -4, -5 and -6 mutants) have been characterized previously and are host-range mutants that replicate well in human HeLa cells but not in mouse cells. Two deletion mutants, SLII-2 and SLII-3, were non-viable, even in HeLa cells. It is now reported that SLII-2 was defective in genome RNA synthesis and viral protein synthesis, while SLII-3 was defective only in viral protein synthesis. These results indicate that the SLII region contains a *cis*-element for RNA replication as well as for IRES-dependent translation and that these two functions lie at the same sites within the SLII region. The host cellular factors that interacted with wild-type SLII and mutant SLII-2 and SLII-3 RNAs were different, suggesting that different host-factor binding regulates expression of mutant phenotypes.

The genome of poliovirus is a single-stranded, positive RNA composed of approximately 7500 nucleotides, to which a small protein, VPg, is attached at the 5' end (Lee *et al.*, 1977; Nomoto *et al.*, 1977) and which has a poly(A) tract at the 3' end (Yogo & Wimmer, 1972). The genome RNA itself functions as mRNA and contains a long, highly structured 5' non-coding region (NCR) of 742 nucleotides in poliovirus type 1. Translation is initiated by entry of ribosomes into the internal ribosome entry site (IRES) (Jang *et al.*, 1988) within the 5' NCR (Sonenberg, 1989). Six stem-loop (SL) structures (SLI, II, III, IV, V and VI) were predicted within the 5' NCR (Wimmer *et al.*, 1993). SLI, a possible cloverleaf-like structure, is thought to be required for the initiation of virus RNA replication, and SLII, IV, V and part of VI are thought to be required for virus

internal translation initiation (Meerovitch *et al.*, 1991; Nicholson *et al.*, 1991; Wimmer *et al.*, 1993).

Six poliovirus mutants were constructed by introducing mutations into the SLII region, and were named SLII-1 to -6. Four of these mutants, SLII-1, -4, -5 and -6, proliferated well in human HeLa cells but not in mouse TgSVA cells, because of defective translation initiation in mouse cells (Shiroki *et al.*, 1997). The other two mutants (SLII-2 and -3) showed no proliferation even in HeLa cells. In this report, these two non-viable mutants were characterized.

We introduced mutations into nt 128–134 of the wild-type (WT) genome by using a site-directed mutagenesis technique as described previously (Shiroki *et al.*, 1997), generating SLII-2, with a deletion of nt 143–148, and SLII-3, with deletions of nt 137–141 and 150–154 (Fig. 1*a*). Infectious cDNAs of these two mutants and WT, as a control, were transcribed *in vitro* with T7 RNA polymerase. HeLa cells transfected with these RNAs were observed for cytopathic effect until 6 or 7 days after transfection, when most cells were rounded and detached. Poliovirus was recovered from the cells transfected with WT RNA but not from the cells transfected with mutant RNAs (data not shown). Thus, SLII-2 and SLII-3 seemed to be non-viable in HeLa cells.

In order to determine the defective steps in the virus replication cycle of these mutants, the translation and RNA replication activities of these mutants were analysed. Translation ability was analysed in a cell-free translation system by using HeLa S10 extract. As shown in Fig. 1*c*, a protein of 66 kDa was detected in the translation products of WT RNA, but not in the products of SLII-2 and -3 mutant RNAs. This indicates strongly that the IRES activities of both the mutants are defective, resulting in non-viability.

In cells transfected with SLII-2 and -3 mutant RNAs carrying defective IRESs, viral RNA synthesis cannot occur, even if there is an efficient initiation signal for RNA synthesis, because no viral proteins, including RNA polymerase 3D, are produced in transfected cells. To produce virus replication proteins independently of the poliovirus IRES, Molla *et al.* (1992) and Borman *et al.* (1994) used a bicistronic recombinant genome in which an encephalomyocarditis virus (EMCV) IRES was inserted at the junction of poliovirus structural (P1) and non-structural (P2, P3) protein regions. In this system, viral

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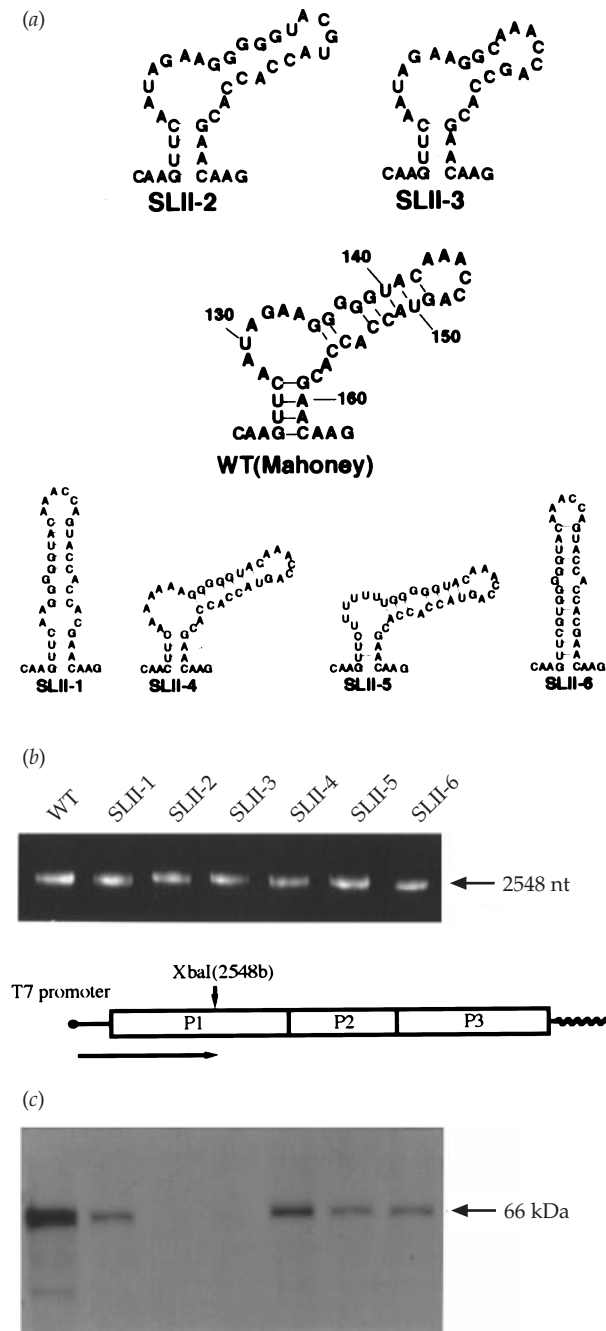


Fig. 1. Possible secondary structures at the site of mutation of SLII mutant genomes and the products of cell-free translation from truncated virus RNAs. (a) Possible secondary structures of the SLII regions of the genomes of WT and mutants are shown. Nucleotide positions are indicated on the nucleotide sequence of the WT genome. (b) Template RNAs with a free 5' end were transcribed by T7 RNA polymerase from cDNAs of WT and mutants that had been cleaved by *Xba*I. The size of template RNA is shown on the right. (c) The *in vitro* transcripts were incubated at 32 °C for 60 min in the S10 fraction, which was prepared from HeLa cells, and treated with micrococcal nuclease, as previously described (Ishii *et al.*, 1998; Shiroki *et al.*, 1997). The translation mixture in sample buffer was heated at 100 °C for 10 min and separated by 10% PAGE. After fixation with 10% acetic acid and 50% methanol, the gels were dried and exposed to X-ray film. The position of the product (66 kDa) is indicated by an arrow.

RNA replication is initiated by RNA polymerase translated from the EMCV IRES even if translation driven by the poliovirus IRES is defective as long as the RNA replication signal is still active. Bicistronic SLII-2, SLII-3 and WT genomes were constructed in a similar manner and designated SLII-2-E, SLII-3-E and WT-E. When these RNAs were transfected to HeLa cells, infectious virions were recovered only from WT-E-transfected cells, because capsid proteins were not translated in the cells transfected with SLII-2-E and SLII-3-E. Virus from WT-E was infectious and RNA replication was reinitiated by secondary infection. Therefore, it is impossible to compare exactly the RNA replication ability of SLII-2-E and SLII-3-E with that of WT-E by measuring the amount of viral RNA extracted from transfected cell cultures. To compare the relative ability for RNA replication of these viruses, an in-frame deletion was introduced into the P1 region (nt 1172–2470), resulting in deletion mutants that act as defective interfering (DI) genomes (Hagino-Yamagishi & Nomoto, 1989), and were designated D-E-SLII-2, D-E-SLII-3 and D-E-WT (Fig. 2*b*). As a control, the same deletion was introduced into SLII-2, SLII-3 and WT and these deletion mutants were designated D-SLII-2, D-SLII-3 and D-WT (Fig. 2). By using these DI genomes, the RNA replication ability of the mutants and WT was assayed (Fig. 2*b*). RNAs of D-WT and D-E-WT, used as a positive control, accumulated in a time-dependent manner, as expected from previously published results (Hagino-Yamagishi & Nomoto, 1989; Borman *et al.*, 1994). There was no accumulation of D-SLII-2 and D-SLII-3 RNA because of defective translation of viral proteins. D-E-SLII-2 RNA appeared not to be synthesized. Since the non-structural proteins must be translated, the SLII-2 mutation is thought also to be defective for RNA replication. D-E-SLII-3 RNA was synthesized, and therefore the SLII-3 mutation was active for viral RNA replication.

The introduction of deletions into the SLII region abolished virus replication. In both SLII-2 and SLII-3 mutants, translational defects blocked virus replication. Of these two mutants, SLII-2 was also defective for viral RNA replication. This indicates that the SLII region contains two *cis*-acting signals, for internal translation (IRES function) and RNA replication. Our previous report suggested that nt 133 within the SLII region is a *cis*-acting signal for RNA synthesis (Shiroki *et al.*, 1995). This paper shows a new site within the SLII region to be a *cis*-element for RNA replication. Poliovirus RNA replication had previously been thought to require only the 5'-proximal region of approximately 100 nucleotides (Andino *et al.*, 1990, 1993). However, Borman *et al.* (1994) showed that there are also *cis*-elements for RNA synthesis in the 3' region of the 5' NCR. These authors showed that a three nt deletion at position 500 reduced both translation efficiency and RNA synthesis, and that disruption of the secondary structure of the IRES around nt 343 reduced viral RNA replication but had minimal effects on IRES function. Both mutations were in loops of the secondary structure of the IRES. The SLII-2 mutation leads to

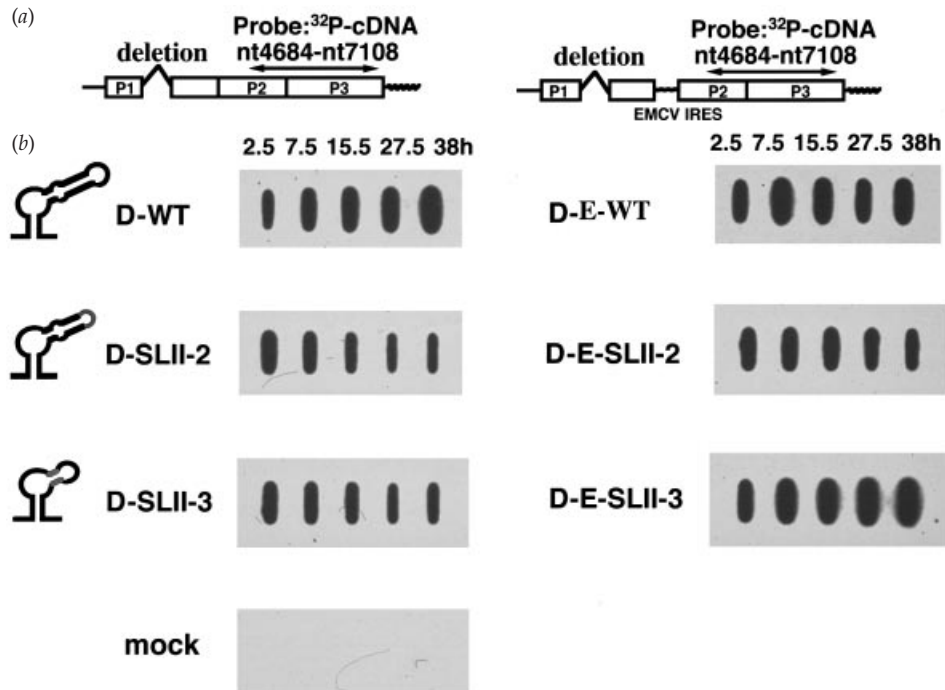


Fig. 2. RNA replication assay. (a) Structures of the RNAs used in this experiment. (b) Subconfluent HeLa monolayer cells in 12-well plates were transfected with 1.5 μ g per well D-WT, D-SLII-2, D-SLII-3, D-E-WT, D-E-SLII-2 or D-E-SLII-3 RNAs by a DEAE-dextran method (Hagino-Yamagaishi & Nomoto, 1989). At the times indicated, cytoplasmic RNAs were extracted from transfected cells and denatured. Almost all of each RNA sample was applied to a slot of a slot-blot apparatus (Manifold II; Schleicher & Schuell) and immobilized on a nylon membrane. The membranes were hybridized to a 32 P-labelled cDNA probe corresponding to nt 4684–7108 of the poliovirus genome.

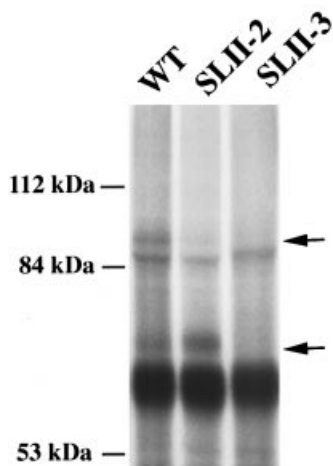


Fig. 3. UV cross-linking assay. 32 P-labelled RNAs (nt 1–730) of WT, SLII-2 and SLII-3 were synthesized from cDNAs that had been linearized by digestion with *MunI* (nt 730). A HeLa S10 extract was fractionated by hydroxyapatite column chromatography (ceramic hydroxyapatite type II, 5 ml; Bio-Rad). Fractions were eluted by a linear gradient of 10–400 mM sodium phosphate buffer (pH 6.8). Each fraction was mixed with the indicated RNAs and UV cross-linked under conditions described previously (Hellen *et al.*, 1993). Fraction 19 (280–300 mM sodium phosphate) is shown. These reaction mixtures were dissolved in sample buffer and applied to SDS-PAGE. After electrophoresis, the gels were dried and autoradiographed. A very prominent band that cross-linked to all RNAs used was identified as polypyrimidine tract-binding protein (data not shown).

disruption of the loop of the SLII region and reduces translation efficiency and RNA synthesis. The SLII-3 mutation, with a deletion of 10 nt, leads to disruption of the stem of the SLII region and reduces only translation efficiency. Thus, nucleotide sequences within the 5' NCR downstream of nt 100 may play some role in RNA synthesis. Lu & Wimmer (1996) showed that the poliovirus IRES can be replaced by that of hepatitis C virus (HCV), which appears to have a totally different structure. It is therefore possible that certain genetic segments of the HCV IRES share some functions with the poliovirus IRES element.

Many host-cell factors are necessary for poliovirus RNA replication or internal translation, including PTB, La protein, Sam68 and PCBP-1 and -2, the last two being required specifically for RNA replication (Andino *et al.*, 1990, 1993; Blyn *et al.*, 1997; Gamarnik & Andino, 1997; Hellen *et al.*, 1993; McBride *et al.*, 1996; Meerovitch *et al.*, 1993; Parsley *et al.*, 1997). Viral and/or host cellular proteins may interact with the SLII region and blockage of this interaction by mutation may result in defective viral RNA replication or translation. To investigate this possibility, a UV cross-linking assay was performed by using 32 P-labelled, positive-strand RNA (nt 1–744) of WT, SLII-2 and SLII-3 as a probe. As shown in Fig. 3, the electrophoretic patterns were different for WT, SLII-2 and SLII-3. A 92 kDa protein bound to WT RNA and a 70 kDa protein bound mainly to SLII-2 RNA. Based on the loss of

translational activity in both mutants and the loss of RNA replication activity in SLII-2, this 92 kDa protein may be an essential factor for poliovirus translation and the 70 kDa protein may be an inhibitor of RNA synthesis. In order to confirm this hypothesis, these 92 and 70 kDa proteins must be characterized.

We are very grateful to S. Sugano for help in performing slot-blot analysis, to S. Kuge for helpful discussions and suggestions and to T. Isoyama for preparation of the figures. We thank Y. Sasaki and K. Iwasaki for expert technical assistance and E. Suzuki and M. Watanabe for help in preparation of the manuscript. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and the Ministry of Health and Welfare of Japan and by funds from the Science and Technology Agency of Japan.

References

- Andino, R., Rieckhof, G. E. & Baltimore, D. (1990).** A functional ribonucleoprotein complex forms around the 5' end of poliovirus RNA. *Cell* **63**, 369–380.
- Andino, R., Rieckhof, G. E., Achacoso, P. L. & Baltimore, D. (1993).** Poliovirus RNA synthesis utilizes an RNP complex formed around the 5' end of viral RNA. *EMBO Journal* **12**, 3587–3598.
- Blyn, L. B., Towner, J. S., Semler, B. L. & Ehrenfeld, E. (1997).** Requirement of poly(rC) binding protein 2 for translation of poliovirus RNA. *Journal of Virology* **71**, 6243–6246.
- Borman, A. M., Deliat, F. G. & Kean, K. M. (1994).** Sequences within the poliovirus internal ribosome entry segment control viral RNA synthesis. *EMBO Journal* **13**, 3149–3157.
- Gamarnik, A. V. & Andino, R. (1997).** Two functional complexes formed by KH domain containing proteins with the 5' noncoding region of poliovirus RNA. *RNA* **3**, 882–892.
- Hagino-Yamagishi, K. & Nomoto, A. (1989).** In vitro construction of poliovirus defective interfering particles. *Journal of Virology* **63**, 5386–5392.
- Hellen, C. U. T., Witherell, G. W., Schmid, M., Shin, S. H., Pestova, T. V., Gil, A. & Wimmer, E. (1993).** A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proceedings of the National Academy of Sciences, USA* **90**, 7642–7646.
- Ishii, T., Shiroki, K., Hong, D.-H., Aoki, T., Ohta, Y., Abe, S., Hashizume, S. & Nomoto, A. (1998).** A new internal ribosomal entry site 5' boundary is required for poliovirus translation initiation in a mouse system. *Journal of Virology* **72**, 2398–2405.
- Jang, S. K., Krüsslich, H.-G., Nicklin, M. J. H., Duke, G. M., Palmenberg, A. C. & Wimmer, E. (1988).** A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of Virology* **62**, 2636–2643.
- Lee, Y. F., Nomoto, A., Detjen, B. M. & Wimmer, E. (1977).** A protein covalently linked to poliovirus genome RNA. *Proceedings of the National Academy of Sciences, USA* **74**, 59–63.
- Lu, H. H. & Wimmer, E. (1996).** Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. *Proceedings of the National Academy of Sciences, USA* **93**, 1412–1417.
- McBride, A. E., Schlegel, A. & Kirkegaard, K. (1996).** Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells. *Proceedings of the National Academy of Sciences, USA* **93**, 2296–2301.
- Meerovitch, K., Nicholson, R. & Sonenberg, N. (1991).** In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5' untranslated region. *Journal of Virology* **65**, 5895–5901.
- Meerovitch, K., Svitkin, Y. V., Lee, H. S., Lejbkowitz, F., Kenan, D. J., Chan, E. K. L., Agol, V. I., Keene, J. D. & Sonenberg, N. (1993).** La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *Journal of Virology* **67**, 3798–3807.
- Molla, A., Jang, S. K., Paul, A. V., Reuer, Q. & Wimmer, E. (1992).** Cardiovascular internal ribosomal entry site is functional in a genetically engineered dicistronic poliovirus. *Nature* **356**, 255–257.
- Nicholson, R., Pelletier, J., Le, S.-Y. & Sonenberg, N. (1991).** Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. *Journal of Virology* **65**, 5886–5894.
- Nomoto, A., Detjen, B., Pozzatti, R. & Wimmer, E. (1977).** The location of the polio genome protein in viral RNAs and its implication for RNA synthesis. *Nature* **268**, 208–213.
- Parsley, T. B., Towner, J. S., Blyn, L. B., Ehrenfeld, E. & Semler, B. L. (1997).** Poly (rC) binding protein 2 forms a ternary complex with the 5'-terminal sequences of poliovirus RNA and the viral 3CD proteinase. *RNA* **3**, 1124–1134.
- Shiroki, K., Ishii, T., Aoki, T., Kobashi, M., Ohka, S. & Nomoto, A. (1995).** A new *cis*-acting element for RNA replication within the 5' noncoding region of poliovirus type 1 RNA. *Journal of Virology* **69**, 6825–6832.
- Shiroki, K., Ishii, T., Aoki, T., Ota, Y., Yang, W.-X., Komatsu, T., Ami, Y., Arita, M., Abe, S., Hashizume, S. & Nomoto, A. (1997).** Host range phenotype induced by mutations in the internal ribosomal entry site of poliovirus RNA. *Journal of Virology* **71**, 1–8.
- Sonenberg, N. (1989).** Regulation of translation by poliovirus. *Advances in Virus Research* **33**, 175–204.
- Wimmer, E., Hellen, C. U. T. & Cao, X. (1993).** Genetics of poliovirus. *Annual Review of Genetics* **27**, 353–435.
- Yogo, Y. & Wimmer, E. (1972).** Polyadenylic acid at the 3'-terminus of poliovirus RNA. *Proceedings of the National Academy of Sciences, USA* **69**, 1877–1882.

Received 13 August 1998; Accepted 28 November 1998