JOURNAL OF VIROLOGY, Jan. 1997, p. 1–8 Vol. 71, No. 1
0022-538X/97/$04.00+0

Host Range Phenotype Induced by Mutations in the Internal Ribosomal Entry Site of Poliovirus RNA

KAZUKO SHIROKI,[1]* TOSHIHIKO ISHII,[1] TAKAHIRO AOKI,[1] YOSHIHIRO OTA,[2] WEI-XING YANG,[3] TOSHIHIKO KOMATSU,[4] YASUSHI AMI,[4] MINEO ARITA,[4] SHINOBU ABE,[2] SO HASHIZUME,[2] AND AKIO NOMOTO[1]

Department of Microbiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo, 108,[1] Japan Poliomyelitis Research Institute, Kumegawa-cho, Higashimurayama, Tokyo 189,[2] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyoku, Tokyo 113,[3] and Department of Viral Disease and Vaccine Control, National Institute of Health, 4-7-1 Gakuen, Musashimurayama, Tokyo 208,[4] Japan

Received 17 June 1996/Accepted 18 September 1996

Most poliovirus strains infect only primates. The host range (HR) of poliovirus is thought to be primarily determined by a cell surface molecule that functions as poliovirus receptor (PVR), since it has been shown that transgenic mice are made poliovirus sensitive by introducing the human PVR gene into the genome. The relative levels of neurovirulence of polioviruses tested in these transgenic mice were shown to correlate well with the levels tested in monkeys (H. Horie et al., J. Virol. 68:681–688, 1994). Mutants of the virulent Mahoney strain of poliovirus have been generated by disruption of nucleotides 128 to 134, at stem-loop II within the 5′ noncoding region, and four of these mutants multiplicated well in human HeLa cells but poorly in mouse TgSVA cells that had been established from the kidney of the poliovirus-sensitive transgenic mouse. Neurovirulence tests using the two animal models revealed that these mutants were strongly attenuated only in tests with the mouse model and were therefore HR mutants. The virus infection cycle in TgSVA cells was restricted by an internal ribosomal entry site (IRES)-dependent initiation process of translation. Viral protein synthesis and the associated block of cellular protein synthesis were not observed in TgSVA cells infected with three of four HR mutants and was evident at only a low level in the remaining mutant. The mutant RNAs were functional in a cell-free protein synthesis system from HeLa cells but not in those from TgSVA and mouse neuroblastoma NS20Y cells. These results suggest that host factor(s) affecting IRES-dependent translation of poliovirus differ between human and mouse cells and that the mutant IRES constructs detect species differences in such host factor(s). The IRES could potentially be a host range determinant for poliovirus infection.

Humans are the only natural hosts of poliovirus. Monkeys are also highly susceptible to poliovirus when they are inoculated with virus directly into the central nervous system (CNS). Other animal species are generally not susceptible to the virus. This characteristic species specificity of poliovirus has meant that monkeys have been used as the only animal model for the study of poliovirus neurovirulence and safety testing of oral poliovirus vaccines. Recently transgenic (Tg) mice that are susceptible to all three poliovirus serotypes have been generated by introducing the human gene for the poliovirus receptor (PVR) into the mouse genome (21, 33). Cumulative evidence indicates that the Tg mice are a good second animal model for poliomyelitis (1, 14). These observations demonstrate that PVR is one of the most important determinants of host specificity of the virus. The interaction between receptor proteins and the viral capsid proteins appears to provide determination of the host range (HR) of poliovirus. Mutations associated with mouse-adapted phenotype of poliovirus have been mapped to the region that encodes the viral capsid proteins (23, 28, 29). Although the virus replication involves numerous interactions between viral and host factors, no HR mutant associated with mutations in the region other than the viral capsid protein region has ever been reported.

The genome of poliovirus is a single-stranded, positive-stranded RNA composed of approximately 7,500 nucleotides (nt), polyadenylylated, and covalently linked at the 5′ end to a small protein, VPg (31, 40, 43). The RNA alone is infectious; cells transfected with the RNA produce progeny infectious virion particles. A long 5′ noncoding region (NCR) of approximately 750 nt of the RNA harbors *cis* elements for viral RNA and protein syntheses. A possible cloverleaf-like structure formed by the 5′-proximal end of the RNA (approximately 90 nt) is probably a *cis* element that regulates the synthesis of the plus-strand RNA (2). A 500-nt region immediately downstream of this contains a *cis*-acting element termed the internal ribosomal entry site (IRES), which directs viral translation initiation in a 5′-end- and cap-independent manner (24, 30, 32). This region of the RNA is considered to have a complicated highly ordered structure formed by a number of secondary structures (9, 38).

A number of host cellular factors are required for the expression of IRES function. The translation of poliovirus does not occur in a cell-free translation system prepared from wheat germ and occurs inefficiently and usually incorrectly in rabbit reticulocyte lysates (RRL) (6). The poor translation in RRL, however, is markedly improved by the addition of factors from HeLa cells (7, 16, 32). Other IRESs, such as the IRESs of encephalomyocarditis (EMC) virus RNA (17) and hepatitis C virus RNA (41), are highly expressed in the RRL system, indicating that quantitative and/or qualitative differences in host factors might be needed for the expression of specific IRESs with different structures. This led us to the hypothesis that the host factor requirements of IRES may be altered by the introduction of mutations that affect the IRES structure.

* Corresponding author. Phone: 81-3-5449-5501. Fax: 81-3-5449-5408.

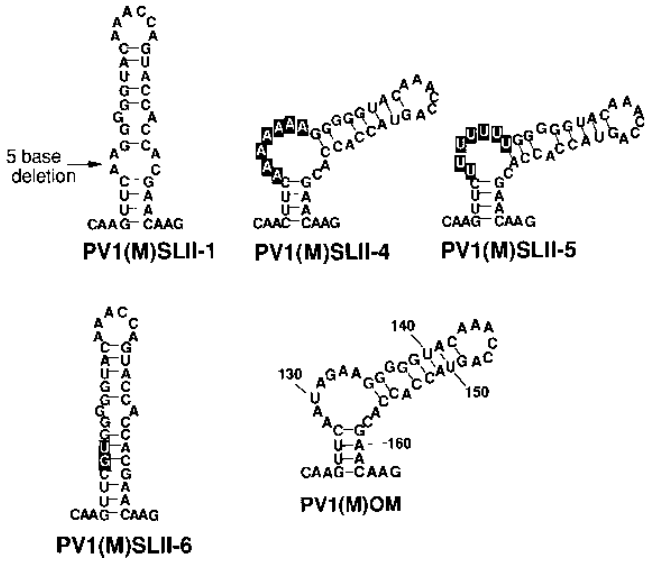

FIG. 1. Secondary structures at the sites of mutation of SLII mutant genomes. Possible secondary structures of SLII regions of the genomes of WT virus and SLII mutants are shown. Nucleotide positions are indicated on the nucleotide sequence of the WT genome.

We have shown previously that plus-strand RNA synthesis of the Mahoney strain of poliovirus type 1 is restricted at an initiation step at 40°C in mouse cells expressing human PVR but not in human HeLa cells (36). Genetic analysis of temperature-resistant (Tr; previously termed Hr) mutants derived from the Mahoney strain identified the key mutation at nt 133 (A to G) within the 5′ NCR of the RNA (37). This nucleotide position is located within the stem-loop II (SLII) structure (nt 126 to 162) and has been considered to be a part of the IRES (24). The data suggest that the SLII region regulates both poliovirus RNA synthesis and IRES-dependent protein synthesis. Borman et al. have suggested that the RNA segment (in the region from nt 343 to 500) within the IRES also controls viral RNA synthesis (7). These observations indicate that the regulatory elements for RNA synthesis and protein synthesis may overlap in part in the 5′ NCR.

To clarify the function of the SLII region, several mutants altered within this region were constructed by using an infectious cDNA clone of the virulent Mahoney strain of poliovirus type 1. The mutants replicated well in primate cells and in the CNS of monkeys but failed to replicate well in mouse cells and in the CNS of the Tg mice. The replication of the mutant strains in mouse cells was blocked at IRES-dependent translation initiation. Mutants retained the capacity for RNA replication in mouse cells. These results suggest that the interaction between the IRES and host factors is an important determinant of host specificity of poliovirus replication.

MATERIALS AND METHODS

Cells and viruses. Suspension-cultured HeLa S3 cells were grown in RPMI 1640 medium supplemented with 5% newborn calf serum and used for preparation of poliovirus type 1 Mahoney strain PV1(M)OM (37) and plaque formation. African green monkey kidney (AGMK) cells cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 5% newborn calf serum were used for plaque formation or transfection with infectious poliovirus cDNAs. TgSVA (36) or NS20Y (established from mouse neuroblastoma) cells were cultured in DMEM supplemented with 5 or 10% fetal calf serum, respectively.

SLII mutants were prepared in AGMK cells at 37°C. Titers of poliovirus preparations were measured by plaque titration in AGMK cells and by cytopathic effect on primary cynomolgus monkey kidney cells. The attenuated Sabin 1 strain used in this study was virus PV1(Sab)IC-0 recovered from AGMK cells transfected with pVS(1)IC-0(T) (19).

Virus infection and growth. To examine virus growth, cells grown in a 12-well plastic culture plate were infected with poliovirus at a multiplicity of infection (MOI) of 20. After incubation for 1 h, the cell cultures were washed with DMEM and treated with neutralizing antibodies against poliovirus for 1 h in order to inactivate unadsorbed virus. Virus yields at appropriate times postinfection were measured by plaque titration in AGMK cells.

Construction of mutant cDNAs. Modifications at nt 128 to 134 of the genome were carried out by two cycles of PCR as described previously (37). Plasmid pT7-1131 derived from PV1(M)OM was used as a template. The first set of primers had a sense nucleotide sequence of nt 126 to 146 and an antisense sequence of nt 389 to 407, and the second set of primers had a nucleotide sequence of pBR vector (nt 3670 to 3690) and an antisense sequence of nt 126 to 146. The sense and antisense primers corresponding to nt 126 to 146 were designed to have mutated sequences that included a five-base deletion from nt 129 to 133 for pOM1-SLII-1, a two-base replacement at U-130 and G-132 by A for pOM1-SLII-4, a seven-base replacement from nt 128 to 134 by seven U's for pOM1-SLII-5, and a two-base change at A-128 and A-129 of pOM1-SLII-1 by G and U, respectively, for pOM1-SLII-6. Two fragments produced by the first and second rounds of PCRs were ligated by further PCR with primers with a sense sequence of nt 3670 to 3690 and an antisense sequence of nt 389 to 407. The *Pvu*I and *Pin*AI fragments from the mutant fragments were inserted into the *Pvu*I and *Pin*AI site of plasmid pOM1. Infectious cDNAs thus constructed were designated pOM1-SLII-1, pOM1-SLII-4, pOM1-SLII-5, and pOM1-SLII-6 (Fig. 1). Viruses produced in AGMK cells transfected with viral RNAs from these mutant cDNAs were designated PV1(M)SLII-1, PV1(M)SLII-4, PV1(M)SLII-5, and PV1(M)SLII-6, respectively (Table 1). Nucleotide sequences of nt 1 to 339 of cDNAs were determined to confirm the modified sequences by a dideoxy method using a Pharmacia LKB ALFred DNA sequencer. Plasmid pOM1-E was pOM1 that carried the EMC virus IRES (EMC virus nt 305 to 845 from plasmid p-CITE [Novagen]) at the junction of the RNA regions encoding P1 and P2 proteins. The structure of pOM1-E was that reported by Borman et al. (7). Plasmids pOM1-E-SLII-1, pOM1-E-SLII-4, pOM1-E-SLII-5, and pOM1-E-SLII-6 were constructed by a similar method.

Tg mouse neurovirulence tests. Tg mice, line IQI-PVRTg21 (heterozygote) (1, 21), 6 to 7 weeks old were inoculated intracerebrally with 30 μl or intraspinally with 5 μl of poliovirus suspensions at various titers. The mouse line had been maintained under specific-pathogen-free conditions before use. Animals were observed every 12 h for paralysis or death up to 14 days after inoculation. In some cases, viruses were isolated from the spinal cords and brains of paralyzed mice.

Monkey neurovirulence tests. Monkey neurovirulence tests were carried out as described previously (18). Cynomolgus monkeys were inoculated with 0.1 ml of a virus suspension (10^7 50% cell culture infective doses per ml) into the lumbar enlargement. Monkeys showing severe clinical poliomyelitis were sacrificed at the peak of disease. Lesion scores were determined by an established procedure to evaluate the intensity of histological lesions (42).

Analysis of proteins by PAGE. Proteins in infected cells were analyzed by polyacrylamide gel electrophoresis (PAGE) as described previously (36). HeLa S3 and TgSVA cells in 12-well plates were infected with poliovirus at an MOI of 50. The cells were incubated in methionine-free DMEM and labeled with 2 mCi of [^{35}S]methionine (1.45 Ci/mmol; Amersham) per ml for 40 min prior to harvest. At intervals of 1 h, the radiolabeled cell monolayers were collected and dissolved in a sample buffer. Samples were heated at 100°C for 5 min, applied to 12.5% polyacrylamide gel, and run at 40 mA. After treatment with Amplify (Amersham), the gels were dried and exposed to Fuji X-ray films.

Cell-free translation. Cytoplasmic extracts (S10) from suspension cultured HeLa cells uninfected or infected with poliovirus were prepared and treated with micrococcal nuclease as previously reported (15). Mouse TgSVA and NS20Y

TABLE 1. Nomenclature of viruses and their infectious cDNA clones

Virus	cDNA clone
WT (Mahoney)	
PV1(M)OM	pOM1
SLII mutants (HR mutants)	
PV1(M)SLII-1	pOM1-SLII-1
PV1(M)SLII-4	pOM1-SLII-4
PV1(M)SLII-5	pOM1-SLII-5
PV1(M)SLII-6	pOM1-SLII-6
PVE mutants (dicistronic viruses)	
PVE	pOM1-E
PVE-SLII-1	pOM1-E-SLII-1
PVE-SLII-4	pOM1-E-SLII-4
PVE-SLII-5	pOM1-E-SLII-5
PVE-SLII-6	pOM1-E-SLII-6

TABLE 2. Plaque-forming abilities of SLII mutants

Virus	PFU/ml		
	HeLa	AGMK	TgSVA
PV1(M)OM (WT)	8×10^9	10^9	4×10^9
PV1(M)SLII-1	1.5×10^9	2×10^8	$\leqq 10^2$
PV1(M)SLII-4	10^9	1.5×10^8	10^5
PV1(M)SLII-5	4×10^8	3×10^8	$\leqq 10^2$
PV1(M)SLII-6	6×10^8	3×10^7	$\leqq 10^2$

monolayer cells were collected and cultured in suspension for 4 and 6 h, respectively. Then the suspension-cultured TgSVA cells were infected with poliovirus and incubated further for 4 h. The suspension-cultured NS20Y cells were used directly for preparation of S10 extracts. Cytoplasmic extracts (S10) were prepared from these mouse cells by the method used for HeLa cells. Conditions for the translation reaction were as specified by Iizuka et al. (15). After incubation at 32°C for 1 h, radioactive products in the translation mixtures were analyzed by sodium dodecyl sulfate-PAGE as described above.

Slot blot analysis. HeLa monolayer cells in 12-well plates were infected with the wild type (WT), SLII mutants, and PVE-WT (see below), and PVE-SLII mutants at an MOI of 5. At indicated times, cytoplasmic RNAs were extracted from the infected cells and denatured. In each slot of a slot-blotting apparatus (Minifold II; Schleicher & Schuell), 20 mg of RNA was applied, and the RNAs were immobilized on a nylon filter (Hybond N; Amersham). The filters were hybridized to a ^{32}P-labeled cDNA corresponding to nt 4684 to 7108 of the viral RNA.

RESULTS

Characterization of poliovirus mutants. Poliovirus mutants disrupted between nt 128 to 134 in the SLII (SLII mutants) were constructed by PCR site-directed mutagenesis of an infectious cDNA clone of the Mahoney strain of poliovirus type 1 as described in Materials and Methods and were designated PV1(M)SLII-1, PV1(M)SLII-4, PV1(M)SLII-5, and PV1(M)SLII-6. The structures of the sites of mutation are shown in Fig. 1.

Virus preparations of the SLII mutants were grown in primate (HeLa and AGMK) cells and TgSVA cells at 37°C (Table 2). All SLII mutants show fairly high titers in the primate cells. In HeLa and AGMK cells, the SLII mutants displayed plaques at 10^{-8} and 10^{-7} dilutions, respectively. However, plaques were not observed in mouse TgSVA cells infected even at a 10^{-2} dilution of the PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 preparations. The PV1(M)SLII-4 mutant also showed a significantly lower titer in TgSVA cells than in primate cells. The virus titers of WT poliovirus in TgSVA cells and primate cells are similar. These observations suggest that the SLII mutants retain an almost complete ability to replicate in primate cells but replicate very poorly in mouse TgSVA cells. SLII mutants formed smaller plaques in primate cells than WT poliovirus (data not shown). This result may suggest that the SLII mutants multiply with a slightly lower efficiency in the primate cells than WT poliovirus.

To determine the virus yield in a single cycle of infection, HeLa and TgSVA cells were infected with WT poliovirus or the SLII mutants at an MOI of 20, and the time course of virus production after infection was followed (Fig. 2). In HeLa cells, the virus production of WT poliovirus plateaued 7 h after infection, and those of the SLII mutants plateaued 13 h after infection. Maximum yields of the SLII mutants in HeLa cells were slightly lower (1/5 to 1/10) than that of WT poliovirus. In TgSVA cells, however, yields of WT and the SLII mutants were significantly different. WT poliovirus replicated to a similar level in TgSVA cells as in HeLa cells. However, the yields of PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 were 10^4- to 10^5-fold lower and the yield of PV1(M)SLII-4 was 10^2- to 10^3-fold lower in TgSVA cells than in HeLa cells. Thus, SLII mutants have a very poor capacity to replicate in mouse TgSVA cells. Since a similar phenomenon was observed when mouse Lα cells (20) were used as host cells (data not shown), it is possible that the SLII mutants constructed here have HR-dependent replication.

Neurovirulence of mutants in Tg mice and monkeys. The neurovirulence of the SLII mutants was tested in the Tg mice by intracerebral inoculation (Fig. 3). All Tg mice inoculated with 10^3 PFU or more of the virulent Mahoney strain died, whereas no mice died following inoculation of up to $10^{6.5}$ PFU of the attenuated Sabin 1 strain. The Sabin 1 strain failed to even cause paralysis in the Tg mice. None of the mice inoculated with PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 died, although a few of the mice inoculated with PV1(M)SLII-1 and PV1(M)SLII-5 had evidence of paralysis. These observations suggested that the levels of neurovirulence in

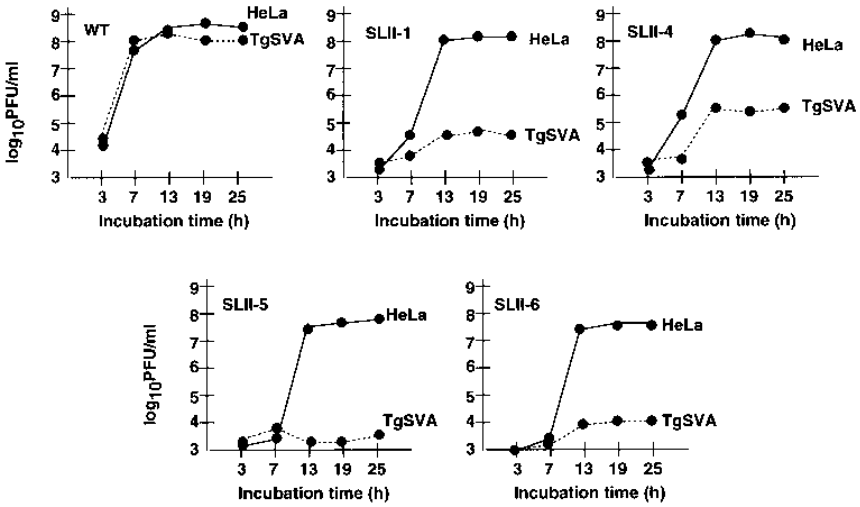

FIG. 2. Virus yield in HeLa and TgSVA cells in a single cycle of infection. HeLa and TgSVA cells in 12-well plates were infected with viruses at an MOI of 20 and cultured at 37°C. At indicated times, viruses were recovered from cells by freeze and thawing. Virus yields were measured by plaque assay on AGMK cells. PV1(M) is omitted from nomenclatures of SLII mutants.

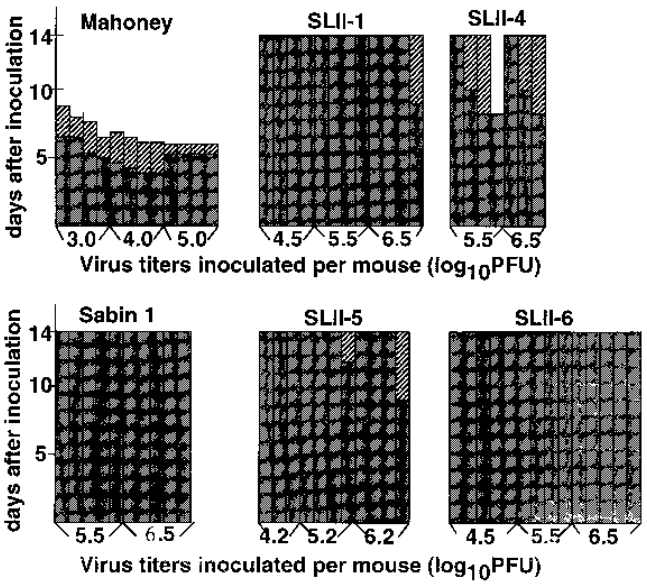

FIG. 3. Neurovirulence tests on SLII mutants in Tg mice by the intracerebral route. Tg mice were inoculated intracerebrally with the Mahoney strain, the Sabin 1 strain, or the PV1(M)SLII-1, -4, -5, and -6 strains and observed for paralysis and death up to 14 days after inoculation. Lengths of bars represent survival times of individual Tg mice. The shadowed bars and hatched bars indicate survivors without any clinical signs and with paralysis, respectively.

mice of PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 were similar to that of the attenuated Sabin 1 strain and were significantly lower than that of the parental virulent Mahoney strain. One of four mice inoculated with $10^{5.5}$ PFU of PV1 (M)SLII-4 died during the test. The incidence of paralysis was higher in the mice inoculated with PV1(M)SLII-4 than in those inoculated with other SLII mutants. The data suggested that PV1(M)SLII-4 had a slightly higher neurovirulence than the other SLII mutants, although the neurovirulence of this mutant remained significantly lower than that of the parental Mahoney strain. Intraspinal mouse neurovirulence tests on PV1(M)SLII-1 and PV1(M)SLII-6 also demonstrated that these SLII mutants had neurovirulence levels similar to that of the attenuated Sabin 1 strain (data not shown).

Monkey neurovirulence tests with intraspinal inoculation were carried out with the PV1(M)SLII-1 strain as described previously (18) (Table 3). All three monkeys inoculated with 10^6 50% cell culture infective doses of PV1(M)SLII-1 showed severe paralysis, and two of them died within 7 days after the inoculation. In contrast, the attenuated Sabin 1 strain caused mild paralysis in only 4 of 12 monkeys inoculated, and no monkeys died. Histopathological examinations were carried out on the CNS of infected monkeys. Mean lesion scores obtained were 2.16 and 0.88 for PV1(M)SLII-1 and the Sabin 1 strain, respectively (Table 3). Since two of three monkeys inoculated died, the mean lesion score due to the PV1(M)SLII-1 strain may have already plateaued in this test (see reference 14). This finding suggests that the intraspinal inoculation

TABLE 3. Monkey neurovirulence test via the intraspinal route

Virus	Incidence of paralysis	Incidence of death	Avg lesion score
PV1(M)SLII-1	3/3	2/3	2.16
Sabin 1	4/12	0/12	0.88

TABLE 4. Plaque-forming abilities of viruses isolated from paralyzed animals

Virus	PFU/ml	
	TgSVA	AGMK
PV1(M)OM (WT)	10^9	6×10^8
Mouse-1	10^5	10^7
Mouse-2	10^5	5×10^7
Monkey-1	$\leqq 10^2$	7×10^7
Monkey-2	$\leqq 10^2$	8×10^7
PV(M)SLII-1	10^2	7×10^7

method was too sensitive to assess the neurovirulence level of the virus. The results indicate that the SLII mutant had a strong neurovirulent phenotype in monkeys, significantly higher than that of the attenuated Sabin 1 strain.

The flaccid paralysis and death that are characteristic of poliomyelitis are believed to be caused by lytic replication of poliovirus in motor neurons in the anterior (dorsal) horn of the spinal cord and in respiratory neurons in the brain stem, respectively. Therefore, the results of neurovirulence tests using the Tg mice and monkeys suggest that the SLII mutants grow well in neurons of the CNS of monkeys but not in those of the Tg mice, indicating that the SLII mutants described in this study are HR specific.

Characterization of virus passaged in the CNS of Tg mice or monkeys. The high neurovirulence of the SLII mutant in monkey might result from mutations that arise during virus replication in the monkey CNS. To exclude this possibility, virus was recovered from the spinal cord and brain of a paralyzed monkey that had been injected with PV1(M)SLII-1. The recovered virus was grown in AGMK cells, and plaque formation was determined in TgSVA and AGMK cells. None of the recovered virus produced plaques in TgSVA cells. Two examples (monkey-1 and monkey-2) are shown in Table 4. This result suggests that the SLII mutant was not altered in the replication process of monkey CNS and that the SLII mutant itself has a fairly strong neurovirulence. Viruses recovered from the CNS of a paralyzed mouse infected with PV1(M) SLII-1 acquired plaque-forming ability in TgSVA cells (Table 4). This observation suggests that revertants of the SLII mutant may cause paralysis in the Tg mice (Fig. 3). Thus, it is likely that the SLII mutants replicate to a sufficiently high level to cause paralysis and death in the CNS of monkeys but not in the CNS of the Tg mice. These results strongly support the notion that the SLII mutants are HR mutants. The revertants isolated from the CNS of the Tg mice are currently being characterized at the molecular level.

Viral protein synthesis in infected cells. The SLII structure harbors regulatory elements for both plus-strand RNA synthesis and IRES-dependent protein synthesis. It is therefore possible that the mutations introduced in this study may block one or both of these virus replication processes in mouse cells. Viral protein synthesis and a shutoff of host protein synthesis were investigated in HeLa and TgSVA cells infected with the WT strain or the SLII mutants at an MOI of 50 (Fig. 4). At 1-h intervals, a portion of the cells was pulse-labeled with [^{35}S]methionine for 40 min prior to harvest in order to examine the relative amount of protein synthesis. Infection with WT strain resulted in efficient synthesis of poliovirus-specific proteins and strong inhibition of host cell protein synthesis both in HeLa and TgSVA cells (Fig. 4A). Similar results were observed in HeLa cells infected with the SLII mutants (Fig. 4B to E). In TgSVA cells, however, all of the SLII mutants except PV1

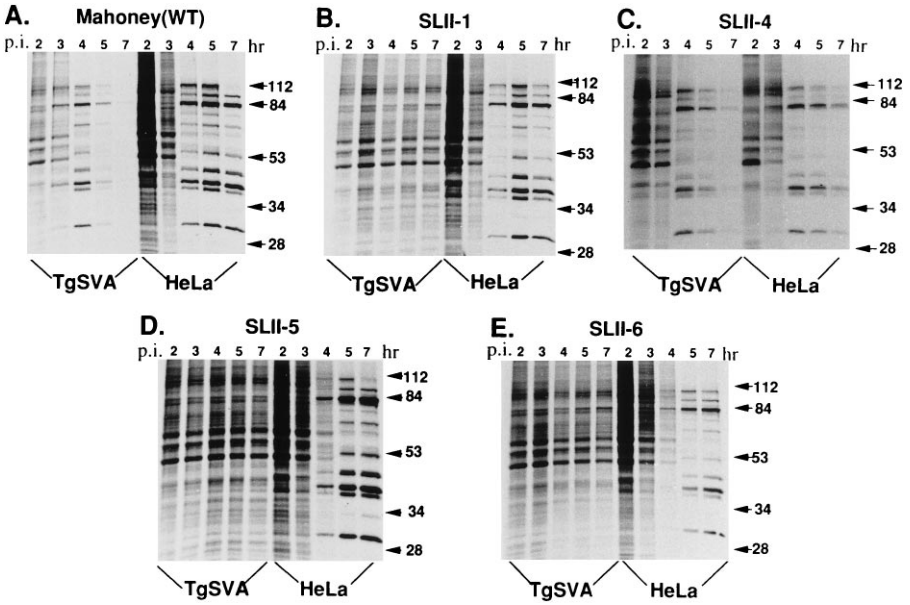

FIG. 4. Protein synthesis in HeLa and TgSVA cells infected with SLII mutants. HeLa and TgSVA cells in 12-well plates were infected with the Mahoney(WT)(A) or PV1(M)SLII-1(B), SLII-4(C), SLII-5(D), or SLII-6(E) strain at an MOI of 50 and labeled with [^{35}S]methionine for 40 min prior to harvest. At 1, 2, 3, 4, 5, and 7 h postinfection (p.i.), extracts were prepared and analyzed as described in Materials and Methods. Sizes (kilodaltons) of protein markers are indicated on the right of each panel.

(M)SLII-4 failed to induce virus-specific protein synthesis and shutoff of cellular protein synthesis (Fig. 4B, D, and E). The PV1(M)SLII-4 strain exhibited both processes in HeLa and TgSVA cells, as was observed with the WT strain (Fig. 4C). Thus, virus-specific translation does not proceed in TgSVA cells infected with the PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 strains. Monitoring the inhibition of host cell protein synthesis is known to be sensitive for the detection of translation of poliovirus proteins directly from the virion RNA early in the infection cycle (36). Therefore, those observations indicate that initiation of translation that is directed by the IRES of virion RNA is blocked in these three mutants in TgSVA cells. The defective replication process of the SLII mutants in TgSVA cells therefore appears to occur at or upstream of IRES-dependent translation.

Cell-free translation. Cell-free translation systems were used for further investigation of the IRES activity of SLII mutant RNAs. RNAs with a free 5′ end were synthesized from cDNA clones pOM1 and pOM1-SLII-1, -4, -5, and -6 that had been linearized by digestion with *Xba*I; these RNAs were used as templates in S10 extracts prepared from HeLa, TgSVA, and NS20Y cells (Fig. 5). The amounts of RNA templates used are shown in Fig. 5D. As expected, a protein band of 66 kDa is detected following translation of all of the RNAs in S10 extracts from HeLa cells (Fig. 5A). The intensities of the bands, however, differ among samples. WT RNA resulted in the largest band, and the SLII mutant RNAs produced relatively small amounts of protein. This is compatible with the observation that HeLa cells infected with the SLII mutants produce progeny infectious particles at a slightly lower level than those infected with WT poliovirus (Fig. 2).

In S10 extracts from mouse cells, the 66-kDa protein was not detected in the products of any of the SLII mutant RNAs but was visible in the products of the WT RNA (Fig. 5B and C). It is possible that the SLII mutant RNAs are unstable and rapidly degraded in S10 extracts from mouse cells. To exclude this possibility, the IRES of EMC virus was inserted at the junction between the genomic regions encoding the capsid (P1) proteins and noncapsid (P2 and P3) proteins of poliovirus (Fig. 6D), and these constructs were used as additional templates for protein synthesis in the S10 extracts. As shown in Fig. 6B and C, bands corresponding to P2 and P3 proteins of poliovirus were seen in every reaction product, although a 66-kDa protein

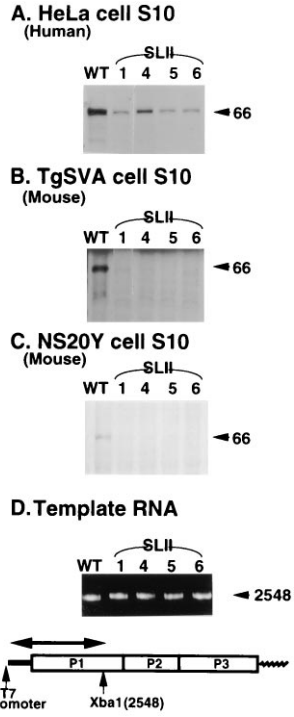

FIG. 5. Cell-free translation products of truncated virus RNAs. Template RNAs were transcribed by T7 RNA polymerase from pOM1 and pOM1-SLII-1, -4, -5, and -6 strains that had been cleaved by *Xba*I (D). The nucleotide length of template RNA is shown on the right of panel D. The in vitro transcripts were incubated for 60 min at 32°C in S10 from HeLa (A), TgSVA (B), and NS20Y (C) cells. Positions of the product (66 kDa) are indicated by arrowheads on the right.

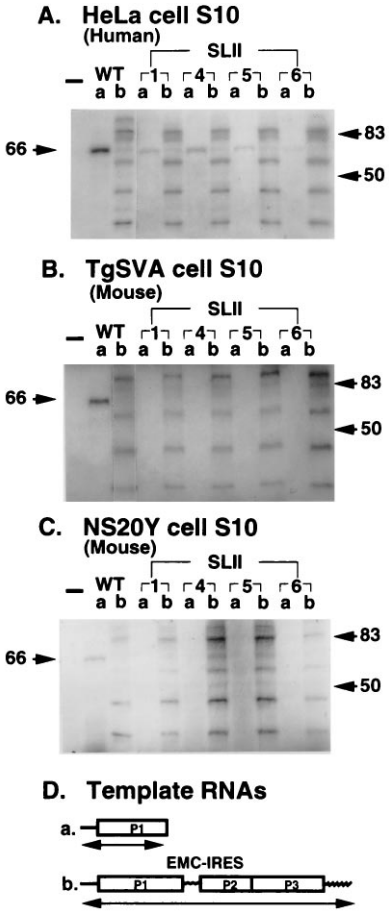

FIG. 6. Cell-free translation products of truncated virus RNAs and PVE mutant RNAs. The template RNAs (D) were transcribed by T7 RNA polymerase from pOM1 and pOM1-SLII-1, -4, -5, and -6 strains that had been cleaved by *Xba*I (a) and pOM1-E, pOM1-E-SLII-1, -4, -5, and -6 strains (b). Lengths of template RNAs are also indicated by lines with arrowheads at both ends in panel D. The in vitro transcripts a and b were translated for 60 min at 32°C in S10 from HeLa (A), TgSVA (B), and NS20Y (C) cells. Sizes (kilodaltons) of protein markers are indicated on the right. Positions of the product (66 kDa) are indicated on the left.

was detected only in translation products from WT RNA. Thus, it is unlikely that only mutant RNAs are unstable in mouse cell extracts. These observations indicate that the IRES of the SLII mutant RNAs does not function in the mouse system but is active in the primate system. These results indicate that the defect(s) of these mutants resides in the IRES-dependent translation initiation in mouse cells.

Viral RNA synthesis. The mutations introduced in this study may also block the SLII function in plus-strand RNA synthesis. To examine this possibility, viruses that had dicistronic RNA genomes carrying the IRES of EMC virus (Fig. 6D) were prepared by transfection of AGMK cells with the corresponding RNAs as described in Materials and Methods. WT virus and the PV1(M)SLII-1, PV1(M)SLII-4, PV1(M)SLII-5, and PV1(M)SLII-6 strains that had the IRES of EMC virus were designated PVE, PVE-SLII-1, PVE-SLII-4, PVE-SLII-5, and PVE-SLII-6 (Table 1). HeLa and TgSVA cells were infected with those dicistronic viruses, and the relative amounts of the RNAs in infected cells were measured over time after infection by slot blot analysis (Fig. 7). As shown in Fig. 6, all of the dicistronic RNAs produced the viral P2 and P3 proteins driven by the inserted IRES from the EMC virus RNA. Since only the viral capsid proteins are dispensable for RNA replicon activity of poliovirus (10), it was expected that the SLII mutant RNAs would show an RNA replicon activity with the aid of the foreign IRES if a *cis* element in SLII structure was still active in RNA synthesis.

As shown in Fig. 7, the amounts of viral RNAs of PVE, PVE-SLII-1, PVE-SLII-5, and PVE-SLII-6 were similar to those of the corresponding parental viruses in HeLa cells. In TgSVA cells, RNA of PVE accumulated to significant levels as did WT RNA. As expected, RNAs of the PV1(M)SLII-1, PV1(M)SLII-5, and PV1(M)SLII-6 strains appeared not to accumulate in TgSVA cells. However, a considerable amount of RNAs accumulate in TgSVA cells infected with the PVE-SLII-1, PVE-SLII-5, and PVE-SLII-6 strains. These experiments demonstrate that the *cis* element of the SLII structure for RNA synthesis is active in these SLII mutant RNAs.

DISCUSSION

Host-restricted expression of poliovirus neurovirulence is thought to be regulated by PVR, a cell surface glycoprotein that belongs to the immunoglobulin superfamily (20, 26). Tg mice carrying the human PVR gene have been shown to be susceptible to all three poliovirus serotypes (21, 33). Furthermore, it has been demonstrated that the relative neurovirulence levels of various poliovirus isolates in monkey and Tg mouse models are correlated well with each other (1, 14). Neurovirulence tests in the two animal models have similarly demonstrated that a relatively strong neurovirulence determinant(s) resides in the IRES of poliovirus type 1 and that nt 480 is particularly important (14, 18). Thus, the IRES seems to be an important regulatory element for strain-specific expression of poliovirus neurovirulence in these two host animals. These two animal models show no difference in the development of the disease, despite the fact that replication of virus in vivo must involve a number of biological interactions between viral and host factors.

Here, we describe the isolation of poliovirus mutants carry-

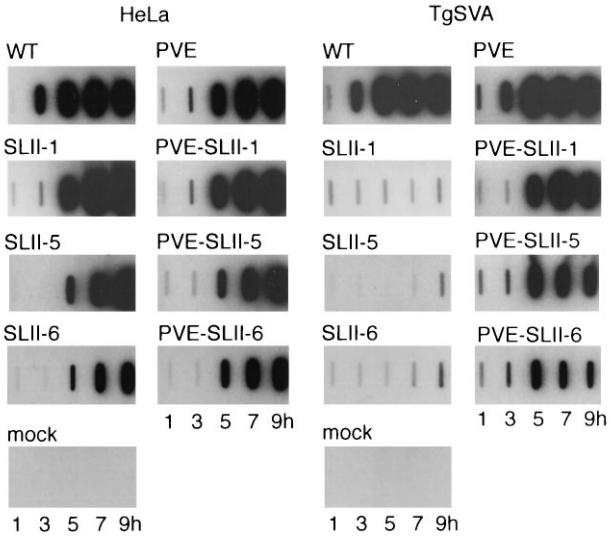

FIG. 7. RNA replication assay. HeLa and TgSVA cells in 12-well plates were infected with WT poliovirus, SLII mutants, and PVE mutants at an MOI of 5. Cytoplasmic RNAs were extracted at indicated times, and amounts of viral RNAs were determined by slot blot analysis as described in Materials and Methods.

ing mutations in the region of nt 128 to 134 within the SLII structure of the RNA. The mutants replicated well in primate cells but not in mouse cells. Furthermore, the mutants showed high neurovirulence in monkeys but low neurovirulence in the Tg mice. The SLII structure has a dual function, playing important roles in the expression of the IRES function (30) and in the initiation of plus-strand RNA synthesis (37). IRES-dependent translation of the SLII mutants was found to be blocked in mouse cells. It was unlikely that the SLII mutants would have defects in other replication processes such as attachment to cells or uncoating of the virion particle, since the mutations were introduced only into the SLII region of the RNA. Our results suggest that interaction of the IRES with host factor(s) is an additional determinant of host-restricted expression of poliovirus neurovirulence and that the SLII mutants constructed here detect a species difference between mice and primates with respect to host factors required for IRES function.

In this study, we used artificial mutants of poliovirus. No naturally occurring poliovirus mutants have been shown to recognize species differences between monkeys and Tg mice (1). It might be possible, however, for HR mutants like the SLII mutants to emerge in nature. If this was the case, the mouse model would fail to assess the relative neurovirulence of the isolates. In addition, the monkey neurovirulence phenotype may not perfectly reflect poliovirus neurovirulence in humans. Tissue-specific replication of the SLII mutants have not been investigated in this study. It is possible that the SLII mutants show tissue-specific replication abilities that are different from that of the WT poliovirus strain, since the distribution of host factors required for the IRES function may differ between cell types.

The initiation events directed by the IRES probably require most of the same set of initiation factors that are utilized by typical capped cellular mRNAs (35). Cap-binding activity of eIF-4F is not required for translation of the uncapped poliovirus RNAs, but the RNA helicase activity manifested by this protein complex may still have an important role in internal initiation (34). If the eukaryotic initiation factor eIF-4F is required for IRES-dependent initiation, it may function in a structurally modified form. It has been shown that eIF-2α associates with nt 97 to 182 and 510 to 629 within the 5′ NCR of poliovirus RNA (3) and that eIF-4B binds to the 5′ NCR of foot-and-mouse disease virus RNA (27). In addition to the standard initiation factors, other *trans*-acting protein factors probably mediate IRES-dependent translation initiation of poliovirus. La protein (p52) and polypyrimidine tract-binding protein (p57) have been reported as host factors for IRES-dependent translation initiation (4, 6, 13, 25, 39). Many unidentified host proteins have been reported to bind to defined IRES regions of poliovirus RNA, using UV cross-linking analysis or gel shift assay (3, 5, 8, 11). Furthermore, viral protein 2A is known to enhance the efficiency of poliovirus translation initiation (12, 22).

Since expression of IRES function must be influenced by efficiency of interactions with host factors, the IRESs of SLII mutants reported here may not be recognized by one or more such host factors. It is unknown at present which host factor(s) is involved in such an interaction, resulting in HR-specific expression of SLII mutant IRESs. To determine the specific regions of the genome involved in the interaction, mutation sites of passaged revertants that can replicate in mouse cells are currently being investigated.

ACKNOWLEDGMENTS

We are grateful to S. Koike and N. Iizuka for critical reading of the manuscript and to S. Kuge and H. Toyoda for helpful suggestions and discussions. We thank H. Chiba, M. Imada, and Y. Sasaki for expert technical assistance and E. Suzuki and N. Masuda 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.

REFERENCES

1. **Abe, S., Y. Ota, Y. Doi, A. Nomoto, T. Nomura, K. M. Chumakov, and S. Hashizume.** 1995. Studies on neurovirulence in poliovirus-sensitive transgenic mice and cynomolgus monkeys for the different temperature-sensitive viruses derived from the Sabin type 3 virus. Virology **210:**160–166.
2. **Andino, R., G. E. Rieckhof, and D. Baltimore.** 1990. A functional ribonucleoprotein complex forms around the 5′-end of poliovirus RNA. Cell **63:**369–380.
3. **Angel, del M., A. G. Papavassiliou, C. Fernandez-Tomas, S. J. Silverstein, and V. R. Racaniello.** 1989. Cell proteins bind to multiple sites within the 5′ untranslated region of poliovirus RNA. Proc. Natl. Acad. Sci. USA **86:**8299–8303.
4. **Belsham, G. J., N. Sonenberg, and Y. V. Svitkin.** 1995. The role of the La autoantigen in internal initiation. Curr. Top. Microbiol. Immunol. **203:**85–93.
5. **Blyn, L. B., R. Chen, B. L. Semler, and E. Ehrenfeld.** 1995. Host cell proteins binding to domain IV of the 5′ noncoding region of poliovirus RNA. J. Virol. **69:**4381–4389.
6. **Borman, A., M. T. Howell, J. G. Patton, and J. R. Jackson.** 1993. The involvement of splicesome component in internal initiation of human rhinovirus RNA translation. J. Gen. Virol. **74:**1775–1788.
7. **Borman, A. M., F. G. Deliat, and K. M. Kean.** 1994. Sequences within the poliovirus internal ribosome entry segment control viral RNA synthesis. EMBO J. **13:**3149–3157.
8. **Dildine, S. L., and B. L. Semler.** 1992. Conservation of RNA-protein interactions among picornaviruses. J. Virol. **66:**4364–4376.
9. **Ehrenfeld, E., and B. L. Semler.** 1995. Anatomy of the poliovirus internal ribosome entry site. Curr. Top. Microbiol. Immunol. **203:**65.
10. **Hagino-Yamagishi, K., S. Kuge, and A. Nomoto.** 1990. Natural and artificial poliovirus defective interfering particles, p. 94–101. *In* M. A. Brinton and F. X. Heinz (ed.), New aspects of positive-strand RNA viruses. American Society for Microbiology, Washington, D.C.
11. **Haller, A. A., and B. L. Semler.** 1995. Stem-loop structure synergy in binding cellular proteins to the 5′ noncoding region of poliovirus RNA. Virology **206:**923–934.
12. **Hambidge, S. J., and P. Sarnow.** 1992. Translational enhancement of the poliovirus 5′ noncoding region mediated by virus-encoded polypeptide 2A. Proc. Natl. Acad. Sci. USA **79:**10272–10276.
13. **Hellen, C. U. T., G. W. Witherell, M. Schmid, S. H. Shin, T. V. Pestova, and E. Wimmer.** 1994. 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. Proc. Natl. Acad. Sci. USA **90:**7642–7646.
14. **Horie, H., S. Koike, T. Kurata, Y. Sato-Yoshida, I. Ise, Y. Ota, S. Abe, K. Hioki, H. Kato, C. Taya, T. Nomura, S. Hashizume, H. Yonekawa, and A. Nomoto.** 1994. Transgenic mice carrying the human poliovirus receptor: new animal model for study of poliovirus neurovirulence. J. Virol. **68:**681–688.
15. **Iizuka, N., H. Yonekawa, and A. Nomoto.** 1991. Nucleotide sequences important for translation initiation of enterovirus RNA. J. Virol. **63:**5354–5363.
16. **Jackson, R. J., M. T. Howell, and A. Kaminski.** 1990. The novel mechanism of initiation of picornavirus RNA translation. Trends Biochem. Sci. **15:**477–488.
17. **Jang, S. K., H.-G. Krausslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62:**2636–2643.
18. **Kawamura, N., M. Kohara, S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1989. Determinants in the 5′ noncoding region of poliovirus Sabin 1 RNA that influence the attenuation phenotype. J. Virol. **63:**1302–1309.
19. **Kohara, M., S. Abe, T. Komatsu, K. Tago, M. Arita, and A. Nomoto.** 1988. A recombinant virus between the Sabin 1 and Sabin 3 vaccine strains of poliovirus as a possible candidate for a new type 3 poliovirus live vaccine strain. J. Virol. **62:**2828–2835.
20. **Koike, S., H. Horie, I. Ise, A. Okitsu, M. Yoshida, N. Iizuka, K. Takeuchi, T. Takegami, and A. Nomoto.** 1990. The poliovirus receptor protein is produced both as membrane-bound and secreted forms. EMBO J. **9:**3217–3224.
21. **Koike, S., C. Taya, T. Kurata, S. Abe, I. Ise, H. Yonekawa, and A. Nomoto.** 1991. Transgenic mice susceptible to poliovirus. Proc. Natl. Acad. Sci. USA **88:**951–955.
22. **Macadam, A. J., G. Ferguson, T. Fleming, D. M. Stone, J. W. Almond, and**

P. D. Minor. 1994. Role for poliovirus protease 2A in cap independent translation. EMBO J. **13:**924–927.
23. **Martin, A., C. Wychowski, T. Couderc, R. Crainic, J. Hogle, and M. Girard.** 1988. Engineering a poliovirus type 2 antigenic site on a type 1 capsid results in a chimeric virus which is neurovirulent for mice. EMBO J. **7:**2839–2847.
24. **Meerovitch, K., R. Nicholson, and N. Sonenberg.** 1991. In vitro mutational analysis of *cis*-acting RNA translational elements within the poliovirus type 2 5′ untranslated region. J. Virol. **65:**5895–5901.
25. **Meerovitch, K., Y. V. Svitkin, H. S. Lee, F. Lejbkowicz, D. J. Kenan, E. K. L. Chan, V. I. Agol, J. D. Keene, and N. Sonenberg.** 1993. La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte. J. Virol. **67:**3798–3807.
26. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56:**855–865.
27. **Meyer, K., A. Petersen, M. Niepmann, and E. Beck.** 1995. Interaction of eukaryotic initiation factor eIF-4B with a picornavirus internal translation initiation site. J. Virol. **69:**2819–2824.
28. **Moss, E. G., and V. R. Racaniello.** 1991. Host range determinants located on the interior of the poliovirus capsid. EMBO J. **10:**1067–1074.
29. **Murray, M. G., J. Bradley, X. F. Yang, E. Wimmer, E. G. Moss, and V. R. Racaniello.** 1988. Poliovirus host range is determined by a short amino acid sequence in neutralization antigen site I. Science **241:**213–215.
30. **Nicholson, R., J. Pelletier, S. Y. Le, and N. Sonenberg.** 1991. Structural and functional analysis of the ribosome landing pad of poliovirus type 2: in vivo translation studies. J. Virol. **65:**5886–5894.
31. **Nomoto, A., B. Detjen, R. Pozzatti, and E. Wimmer.** 1977. The location of the polio genome protein in viral RNAs and its implication for RNA synthesis. Nature **268:**208–213.
32. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature **334:**320–325.
33. **Ren, R., F. Costantini, E. J. Gorgacz, J. J. Lee, and V. R. Racaniello.** 1990. Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. Cell **63:**353–362.
34. **Rozen, F., I. Edery, K. Meerovitch, T. E. Dever, W. C. Merrick, and N. Sonenberg.** 1990. Bidirectional RNA helicase activity of eucaryotic initiation factors 4A and 4F. Mol. Cell. Biol. **10:**1134–1144.
35. **Scheper, G. C., H. O. Voorma, and A. A. M. Thomas.** 1992. Eukaryotic initiation factors-4E and -4F stimulate 5′ cap-dependent as well as internal initiation of protein synthesis. J. Biol. Chem. **267:**7269–7274.
36. **Shiroki, K., H. Kato, S. Koike, T. Odaka, and A. Nomoto.** 1993. Temperature-sensitive mouse cell factors for strand-specific initiation of poliovirus RNA synthesis. J. Virol. **67:**3989–3996.
37. **Shiroki, K., T. Ishii, T. Aoki, M. Kobashi, S. Ohka, and A. Nomoto.** 1995. A new *cis*-acting element for RNA replication within the 5′ noncoding region of poliovirus type 1 RNA. J. Virol. **69:**6825–6832.
38. **Simoes, E. A. F., and P. Sarnow.** 1991. An RNA hairpin at the extreme 5′ end of the poliovirus RNA genome modulates viral translation in human cells. J. Virol. **65:**913–921.
39. **Svitkin, Y. V., K. Meerovitch, H. S. Lee, J. N. Dholakia, D. J. Kenan, V. I. Agol, and N. Sonenberg.** 1994. Internal translation initiation on poliovirus RNA: further characterization of La function in poliovirus translation in vitro. J. Virol. **68:**1544–1550.
40. **Tobin, G. J., D. C. Young, and J. B. Flanegan.** 1989. Self catalyzed linkage of poliovirus terminal protein VPg to poliovirus RNA. Cell **59:**511–519.
41. **Tsukiyama-Kohara, K., N. Iizuka, M. Kohara, and A. Nomoto.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J. Virol. **66:**1476–1483.
42. **World Health Organization.** 1983. Requirements for poliomyelitis vaccine (oral). WHO Expert Committee on Biological Standardization. Annex 4. W. H. O. Tech. Rep. Ser. **687:**130–133.
43. **Yogo, Y., and E. Wimmer.** 1972. Polyadenylic acid at the 3′-terminus of poliovirus RNA. Proc. Natl. Acad. Sci. USA **69:**1877–1882.