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A New *cis*-Acting Element for RNA Replication within the 5′ Noncoding Region of Poliovirus Type 1 RNA

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Mouse cells expressing the human poliovirus receptor (PVR-mouse cells) as well as human HeLa cells are susceptible to poliovirus type 1 Mahoney strain and produce a large amount of progeny virus at 37°C. However, the virus yield is markedly reduced at 40°C in PVR-mouse cells but not in HeLa cells. The reduction in virus yield at 40°C appears to be due to a defective initiation process in positive-strand RNA synthesis (K. Shiroki, H. Kato, S. Koike, T. Odaka, and A. Nomoto, J. Virol. 67:3989–3996, 1993). To gain insight into the molecular mechanisms involved in this detective process, naturally occurring heat-resistant (H^r)-mutants which show normal growth ability in PVR-mouse cells even at 40°C were isolated from a virus stock of the Mahoney strain and their mutation sites that affect the phenotype were identified. The key mutation was a change from adenine (A) to guanine (G) at nucleotide position (nt) 133 within the 5′ noncoding region of the RNA. This mutation also gave an H^r phenotype to the viral plus-strand RNA synthesis in PVR-mouse cells. Mutant Mahoney strains with a single point mutation at nt 133 (A to G, C, or T or deletion) were investigated for their ability to grow in PVR-mouse cells at 40°C. Only the mutant carrying G at nt 133 showed an H^r growth phenotype in PVR-mouse cells. These results suggest that a host cellular factor(s) interacts with an RNA segment around nt 133 of the plus-strand RNA or the corresponding region of the minus-strand RNA, contributing to efficiency of plus-strand RNA synthesis.

The genome of poliovirus is a single-stranded, positive RNA composed of approximately 7,500 nucleotides (nt) to which a small protein, VPg, is attached at the 5′ end (10, 23, 36) and poly(A) is attached at the 3′ end (39). This RNA functions as mRNA to produce a single large precursor polyprotein of 247 kDa after the entry into the host cell cytoplasm. The translation is initiated by entry of ribosomes into the internal sequence (IRES) within the 5′ noncoding region (5′NCR) of the RNA (32). The polyprotein is cotranslationally cleaved by virus-specific proteinases to form viral capsid proteins (VP1, VP2, VP3, and VP4) and noncapsid proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D). Of the viral noncapsid proteins, 2A, 3C, and 3CD are viral proteinases involved in the processing specific to poliovirus polyprotein (17). 2C has ATPase activity (21), 3B is VPg (38), 3D is a viral RNA-dependent RNA polymerase which has unwinding activity (9, 29, 38), 2B and 2C are considered to play important roles in viral RNA synthesis (5, 6, 18, 24, 27), and 3A could have an important role(s) in uridylylation of VPg in the process of initiating RNA synthesis (27, 35, 38).

Poliovirus RNA replication occurs on the membranes in the cytoplasm that are induced by the virus infection (5, 6, 8, 11, 34, 35). Virion RNA functions as a template for synthesis of minus-strand RNA. It is suggested that the initiation of plus-strand RNA synthesis depends on the formation of a ribonucleoprotein complex, possibly formed at the 5′ end of a plus-strand RNA (1, 2, 38), where 3CD (3C and 3D) and a 36-kDa cellular protein bind to a possible cloverleaf-like structure of about 90 5′-proximal nt. The ribonucleoprotein complex may be involved in cleavage of 3AB to form 3B (VPg) and in the formation of VPg-pU(pU) (14). VPg-pU(pU) is considered to act as a primer of plus-strand RNA synthesis mediated by 3D polymerase (1, 34, 38).

Poliovirus must require host cellular factors for its replication cycle in addition to viral proteins (1, 3, 4, 13–15, 20). Most poliovirus strains infect only primates, while other animal species are generally not permissive for poliovirus infection. Nonprimate cells, however, are made permissive for poliovirus infection by introducing the human poliovirus receptor (hPVR) gene or cDNA (15, 20). In fact, a mouse Lα cell line, a subline of mouse L cells expressing hPVR (15), and a transgenic TgSVA cell line that originated from the kidney of a transgenic mouse carrying the hPVR gene (30) are permissive for poliovirus infection, and the infected cells produce a large amount of infectious particles at 37°C. However, replication of the Mahoney strain of poliovirus type 1 is greatly suppressed in these mouse cells at 40°C, whereas virus replication in HeLa cells proceeds normally at 40°C (30). The temperature sensitivity of the viral replication in these mouse cells has been suggested to be due to a defect in the process of initiating plus-strand RNA synthesis if not exclusive (30), suggesting that the mouse cell factor(s) involved in the initiation process is temperature sensitive.

Here we describe the isolation of heat-resistant (H^r) mutants of poliovirus type 1 which show normal growth ability in mouse cells expressing hPVR (PVR-mouse cells) even at 40°C. A key mutation site was found to be located at nt 133 within the 5′NCR of the RNA. The results indicate that the RNA region around nt 133 of plus-strand RNA or the corresponding site of minus-strand RNA is a new *cis*-acting element for RNA replication and that expression of the function(s) of this RNA region may require host cellular factors.

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MATERIALS AND METHODS

Cells, viruses, and viral RNAs. Suspension-cultured HeLa S3 cells were grown in RPMI 1640 medium supplemented with 5% newborn calf serum and used for

TABLE 1. Nomenclature of polioviruses and their infectious cDNA clones

Poliovirus	cDNA clone
WT	
PV1(M)pDS306	pM1(T7)0
PV1(M)OM	pOM1
H^r mutants	
PV1(M)LH1	pM1(T7)LH1
PV1(M)H5	pM2(T7)H5
PV1(M)H7	pM1(T7)H7
PV1(M)AH1	pM1(T7)AH1
Recombinants	
PV1OM(H5)1a	pOM1(H5)1a
PV1OM(H5)1b[a]	pOM1(H5)1b

[a] PV1(M)OM(133G).

virus preparation. African green monkey kidney (AGMK) cells cultured in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum were used for plaque assay or transfection with infectious poliovirus cDNAs. Lα (15), TgSVA (30), $L_{6\text{-}7}$, and $L_{1\text{-}3}$ cells were used for isolation of poliovirus mutants. Lα and TgSVA cells were cultured in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. $L_{6\text{-}7}$ and $L_{1\text{-}3}$ cells are L cells carrying a cosmid DNA which has the promoter of a PVR-mouse homolog and the coding region of the human PVR gene (14a). $L_{6\text{-}7}$ and $L_{1\text{-}3}$ cells were grown in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 400 μM G418.

Virus stocks of poliovirus type 1 Mahoney strain PV1(M)pDS306 (12), which originated from an infectious cDNA clone, pM1(T7)0 (12), were prepared in AGMK cells or HeLa cell suspension cultures and used as wild-type (WT) poliovirus. A new infectious cDNA clone (pOM1) of the Mahoney strain was also used in this study. The virus recovered by transfecting cells with plasmid pOM1 was designated PV1(M)OM. Purifications of viruses and viral RNAs were carried out as described previously (12, 16).

Construction of infectious cDNA clones. Complementary DNAs to the poliovirus genomes were prepared by using Amersham's cDNA Synthesis System Plus. The cDNAs having *Eco*RI adaptors at both ends were inserted into the *Eco*RI site of plasmid vector pSVA14 (12). The cDNAs thus constructed lacked short segments corresponding to the 5′-proximal portion of the genomes. DNAs corresponding to the 5′-proximal region (nt 1 to 1131, *Sph*I site) were prepared by reverse transcriptase PCR from poliovirus RNAs using oligonucleotides 5′-CTGAGAATTCGTAATACGATCACTATAGGTTAAAACAGCTCTGG GGTTG-3′ (nucleotide sequences of the *Eco*RI site, T7 ϕ10 promoter, and nt 1 to 20 of poliovirus RNA) and 3′-CCACCACCTTCAACGGACTA-5′ (antisense sequence of nt 1182 to 1201 of poliovirus RNA) as sense and anti-sense primers, respectively. The PCR product was digested with *Eco*RI and *Sph*I and inserted into the *Eco*RI and *Sph*I sites of pBR322. This construct was termed pT7-1131. To prepare infectious cDNA clones of polioviruses, the PCR products were joined to the remaining cDNAs at the corresponding nt 70/71 (*Kpn*I site). In some cases, the *Aat*II site at nt 1122/1123 was used for joining two cDNAs together to form infectious cDNAs. Viruses produced in cells transfected with infectious cDNA clones pM1(T7)H5 and pM1(T7)LH1 were designated PV1(M)H5 and PV1(M)LH1, respectively (Table 1). The recovered viruses showed H^r phenotypes indistinguishable from those of the viruses used for the molecular cloning.

Construction of recombinant DNAs and mutant cDNAs. Infectious cDNA clones that were recombinants of the WT Mahoney cDNA [pM1(T7)0 or pOM1] and mutant cDNAs [pM1(T7)H5 or pM1(T7)LH1] were constructed by using appropriate cleavage sites of restriction enzymes such as *Pin*AI, *Aat*II, *Nhe*I, and *Bgl*II.

Modifications at nt 133 and 134 of the genome were carried out by two cycles of PCR. The first set of primers have a sense nucleotide sequence of nt 126 to 146 and an antisense sequence of nt 389 to 407, and the second set of primers have a nucleotide sequence of the pBR vector (nt 3670 to 3690) and an antisense sequence of nt 126 to 146. Plasmid pT7-1131 derived from PV1(M)OM was used as a template. The sense primers (nt 126 to 146) were designated to replace A at nt 133 by C or T, and A at nt 134 by G. A cDNA clone in which A at nt 133 was missing was also constructed by using a mutant primer. Two fragments produced by the first and second PCRs were ligated together, and the *Pvu*I and *Pin*AI fragments were inserted into the *Pvu*I and *Pin*AI sites of plasmid pOM1. Infectious mutant cDNAs thus constructed were designated pOM1(133C), pOM1(133T), pOM1(133del), and pOM1(134G). Viruses produced in cells transfected with these mutant cDNAs were designated PV1(M)OM(133G) [or PV1OM(H5)1b] (Table 1), PV1(M)OM(133C), PV1(M)OM(133U), PV1(M)OM(133del), and PV1(M)OM(134G), respectively. 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.

AGMK cells were transfected with cDNAs or their RNA transcripts by the DEAE-dextran method (12). RNA transcripts were synthesized by using the MEGAscript T7 kit on cDNAs which were linearized by digestion with *Pvu*I.

Labeling of infected cells with [^{3}H]uridine. To examine viral RNA synthesis, incorporation of [^{3}H]uridine into viral RNAs was monitored as described previously (30). Briefly, cells in 24-well plates were infected with poliovirus at a multiplicity of infection (MOI) of 20. After a 30-min adsorption period, the cells were incubated at either 37°C or 40°C. At 1.5 h postinfection, actinomycin D (final concentration, 5 μg/ml) and guanidine hydrochloride (final concentration, 1 mM) were added to the cultures. [^{3}H]uridine (final concentration, 1 μCi/ml) was added to the cultures at 2 h postinfection. At indicated times, the cells were collected and trichloroacetic acid-insoluble radioactivities were measured.

RNase protection assay. An RNase protection assay was performed as previously reported by Shiroki et al. (30). Briefly, RNAs were prepared from Lα cells (5×10^7) infected with WT poliovirus or mutants at 5 h postinfection. ^{32}P-labeled probes A and B (see Fig. 6C) were synthesized and used for detection of plus-strand RNA and minus-strand RNA, respectively. Probe A was prepared from plasmid pBL-PVKB, which had been linearized by *Bgl*I digestion, by T3 polymerase (Promega). Probe B was prepared from the same plasmid, which had been linearized by *Spe*I digestion, by T7 polymerase (Promega) (see Fig. 6C). Plasmid pBL-PVKB carries an insert of nt 3665 (*Kpn*I site) to 5601 (*Bgl*II site) of poliovirus cDNA at the *Kpn*I and *Bam*HI sites of the vector (30).

Western blot analysis. Western blot (immunoblot) analysis was performed with a rabbit polyclonal antiserum against C-terminal peptides of 3D protein of poliovirus type 1. Lα cells (10^6) were infected with WT poliovirus, an H^r mutant, or their recombinants at an MOI of 10. After adsorption for 30 min, the infected cells were incubated at 37 or 40°C with or without 1 mM guanidine hydrochloride. At 7 h postinfection, the cells were harvested, and the whole-cell lysates were used as protein samples. The samples were separated by electrophoresis on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel as described previously (30). The proteins in the gel were transferred electrophoretically to a polyvinylidene difluoride filter (Immobilon; Millipore Corp.) in a buffer containing 48 mM Tris-glycine (pH 8.3), 0.037% SDS, and 10% methanol. The filters were blocked with a phosphate-buffered saline solution containing 0.2% Tween 20 (PBS-T) and 5% nonfat dry milk. The blots were incubated with anti-3D serum (1:2,000 dilution) for 60 min at room temperature in PBS-T and washed three times for 10 min each time with PBS-T. The filter was incubated with donkey anti-rabbit antibodies conjugated with horseradish peroxidase (1:2,000 dilution; Amersham Life Science) for 60 min at room temperature and washed three times for 10 min each time as above. The filter was then treated with the ECL detection reagents (Amersham Life Science) for 1 min and subjected to autoradiography for 2 to 5 min.

RESULTS

Isolation of H^r poliovirus mutants. Mutant polioviruses that can grow normally at 40°C in PVR-mouse cells (H^r mutants) were isolated by two different procedures as described below. The first procedure involved 10 cycles to adapt the Mahoney strain of poliovirus type 1, so that mutations occurring during the adaptation process provided the virus strain with the ability to grow normally at 40°C in PVR-mouse cells. One cycle of the adaptation process was as follows. TgSVA and Lα cells were infected with the WT Mahoney virus, PV1(M)pDS306, at an MOI of approximately 0.01 and incubated at 40°C until most cells looked ruined. A small amount of virus present in the cell lysates was grown at 37°C in HeLa cells to prepare a high-titer virus stock. After 10 cycles of the adaptation process, the virus preparations clearly had a cytopathic effect on TgSVA and Lα cells: namely, infected mouse cells were rounded and detached within 1 day after infection. As a result, six H^r munant preparations were independently obtained. From those H^r mutant preparations obtained by passage in TgSVA and Lα cells, PV1 (M)AH1 and PV1(M)LH1 were isolated as H^r mutant strains by three successive plaque purifications in the corresponding PVR-mouse cells at 40°C (Table 1). The second procedure involved isolation of mutants that had already been present in a virus stock of the WT Mahoney virus. $L_{6\text{-}7}$ cells were infected with serial dilutions of the virus and incubated at 37 or 40°C under an agar overlay. The numbers of plaques observed at 40°C were approximately 10^{-4} of those at 37°C. Viruses from single plaques produced at 40°C were examined for their ability to grow in the PVR-mouse cells at 37 and 40°C. The viruses

TABLE 2. Sensitivity of cells to WT Mahoney virus and H^r mutant infection

Cells and °C	Virus titer (PFU/ml)				
	PV1(M) pDS306	PV1(M) AH1	PV1(M) LH1	PV1(M) H5	PV1(M) H7
HeLa					
37	10^9	3×10^9	9×10^8	8×10^8	7×10^8
40	5×10^8	3×10^9	7×10^8	6×10^8	4×10^8
Lα					
37	3.5×10^8	5×10^8	3×10^8	10^8	10^8
40	$<10^4$	5×10^8	2×10^8	7×10^7	4×10^7
TgSVA					
37	10^8	4×10^8	2×10^8	ND[a]	ND
40	$<10^4$	3×10^8	1.5×10^8	ND	ND
L_{6-7}					
37	2×10^8	ND	2.5×10^8	10^8	10^8
40	$<10^4$	ND	10^8	7×10^7	6×10^7

[a] ND, not done.

that showed similar efficiencies of growth at the two temperatures were chosen as mutants. Two H^r mutants derived from different plaques were plaque purified at 40°C three times and designated PV1(M)H5 and PV1(M)H7 (Table 1).

Characterization of H^r mutants. Virus titers of H^r mutants in HeLa, TgSVA, Lα, and L_{6-7} cells at 37 and 40°C were measured to confirm the H^r phenotype of the mutants. The results are summarized in Table 2. Titers of WT Mahoney virus, PV1(M)pDS306, in PVR-mouse cells at 40°C were much lower than those at 37°C, whereas the titers in HeLa cells at both temperatures were essentially the same as those reported previously (30). All H^r mutants showed similar titers at both temperatures in HeLa cells and PVR-mouse cells. The WT Mahoney virus and H^r mutants showed similar plaque sizes in HeLa cells. However, H^r mutants, especially PV1(M)AH1 and PV1(M)LH1, showed larger plaques than the Mahoney virus on PVR-mouse cells (see Fig. 4) (data not shown).

It has been shown that viral positive-strand RNA synthesis is temperature sensitive in PVR-mouse cells infected with the WT Mahoney strain of poliovirus type 1 (30). It is possible therefore that RNA syntheses of H^r mutants isolated as described above are resistant at the elevated temperature of 40°C. To examine this possibility, incorporation of [^{3}H]uridine into poliovirus RNAs was measured at 40°C by using HeLa or Lα cells infected with the WT Mahoney virus or H^r mutants at an MOI of 10 (Fig. 1). Temperature-dependent incorporation of [^{3}H]uridine into viral RNAs was observed in Lα cells infected with the Mahoney strain as reported previously (30). [^{3}H]uridine incorporation by Lα cells infected with H^r mutants, however, was efficient at both 37°C and 40°C. The results suggest that H^r mutants isolated in this study do not have a temperature-sensitive step in the viral RNA synthesis in infected PVR-mouse cells. Thus, H^r mutants appear to be suppressor mutants with respect to a certain defective step(s) in the synthesis of viral RNA of the WT Mahoney virus.

Mutation site influencing the H^r phenotype. To identify a mutation site(s) that influences the H^r phenotype, a molecular genetic approach was employed. Infectious cDNA clones of H^r mutants PV1(M)LH1 and PV1(M)H5 were prepared as described in Materials and Methods and designated pM1(T7) LH1 and pM1(T7)H5, respectively (Table 1). Viruses produced in AGMK cells transfected with those infectious cDNA clones were examined for their growth phenotype in Lα or L_{6-7} cells at 37 and 40°C. The growth ability of viruses recovered from the transfected cells in PVR-mouse cells at both temperatures was the same as those of the viruses used for the molecular cloning (Fig. 2). Accordingly, recombinants of the WT Mahoney virus and H^r mutants were constructed by using infectious cDNA clones of them. Structures of the genomes of the recombinant viruses are shown in Fig. 2.

The recombinant viruses were examined for their growth phenotype in Lα cells at 37 and 40°C (Fig. 2). When the growth phenotypes of recombinant viruses from cDNA clones pM1

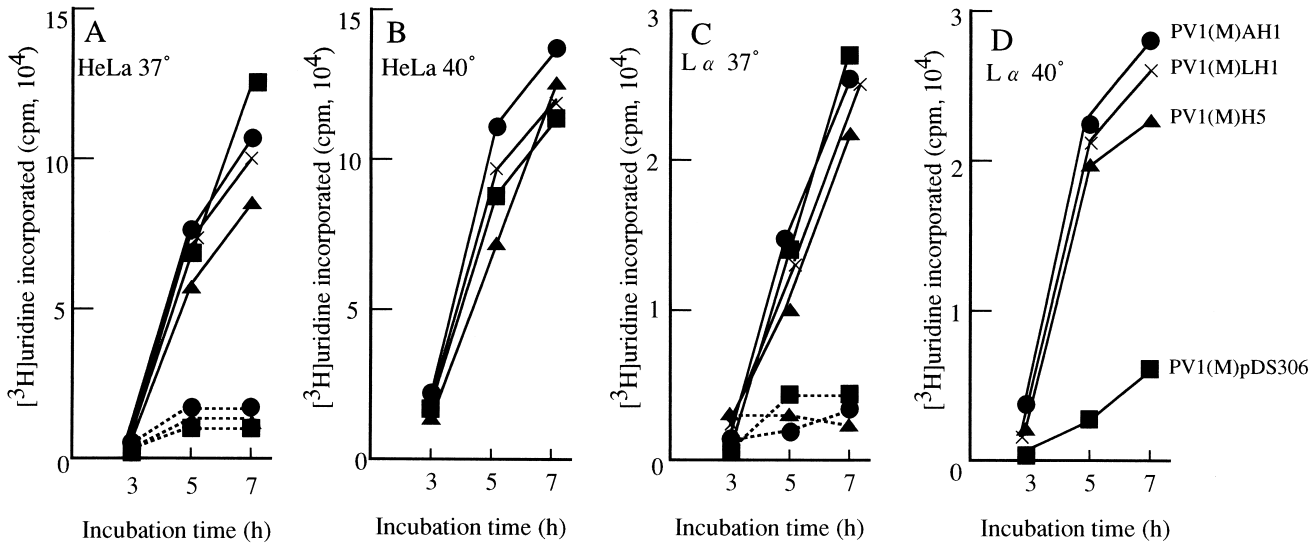

FIG. 1. RNA synthesis of WT poliovirus and H^r mutants. HeLa (A and B) and Lα (C and D) cells in 12-well plates were infected with the WT Mahoney PV1(M)pDS306 virus (■) and H^r mutants PV1(M)AH1 (●), PV1(M)LH1 (×), and PV1(M)H5 (▲) at an MOI of 20 and incubated at 37°C (A and C) or 40°C (B and D). At 1.5 h postinfection, actinomycin D (final concentration, 5 μg/ml) and guanidine hydrochloride (final concentration, 1 mM) (dotted lines) were added. The cultures were then supplemented with 0.1 μCi of [^{3}H]uridine at 2 h after infection. At 3, 5, and 7 h postinfection, cells were collected, and trichloroacetic acid-insoluble counts were measured by a scintillation counter.

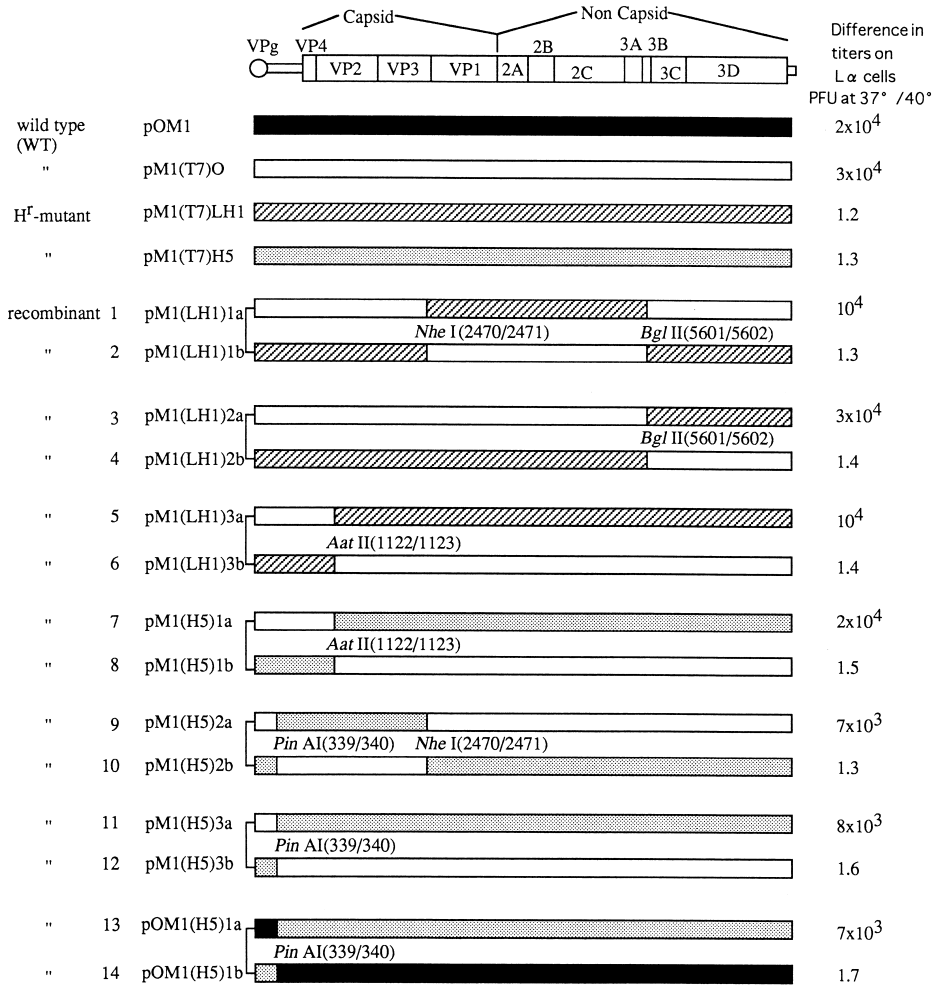

FIG. 2. Genome structures of the recombinant polioviruses and their titers in Lα cells. The genome structures of recombinant viruses are shown as a combination of cDNAs of WT virus [pOM1 and pM1(T7)0] and H^r mutants [pM1(T7)LH1 and pM1(T7)H5]. The *Nhe*I, *Bgl*II, *Aat*II, and *Pin*AI cleavage sites are indicated. Nucleotide positions from the 5′ end of the genome are indicated in parentheses. The genome organization of type 1 poliovirus is shown at the top. The ratios of titers of recombinant viruses at 37°C to those at 40°C in Lα cells are given on the right.

(LH1)1a and pM1(LH1)1b are compared, it is clear that mutations influencing the H^r phenotype are not located in the nucleotide sequence corresponding to the *Nhe*I-to-*Bgl*II fragment (nt 2471 to 5601) (Fig. 2). Similarly, the corresponding RNA sequence downstream of the *Bgl*II site (nt 5602) does not have such a mutation site (compare recombinants 3 and 4 in Fig. 2). These findings indicate that a mutation(s) influencing the H^r phenotype resides in the nucleotide sequence upstream of nt 2470. The recombinant virus from cDNA pM1(LH1)3b (recombinant 6 in Fig. 2), which is composed of the sequence upstream of the *Aat*II site (nt 1122/1123) of pM1(T7)LH1 cDNA (H^r mutant cDNA) and the remaining sequence of pM1 (T7)0 showed the H^r phenotype, and the reciprocal recombinant virus showed a WT Mahoney phenotype. These results strongly suggested that a mutation site(s) responsible for the H^r phenotype resides between nt 1 and 1122 of the genome of H^r mutant PV1(M)LH1. A similar result was obtained when independently isolated H^r mutant PV1(M)H5 was used to construct recombinant viruses (recombinants 7 and 8 in Fig. 2).

Since an H^r mutation site(s) was thought to be located in the region of nt 1 to 1122, nucleotide sequences of the *Aat*II fragments (nt 1 to 1122) from plasmids pOM1, pM1(T7)0, pM1(T7)H5, and pM1(T7)LH1 were determined (Fig. 3). The

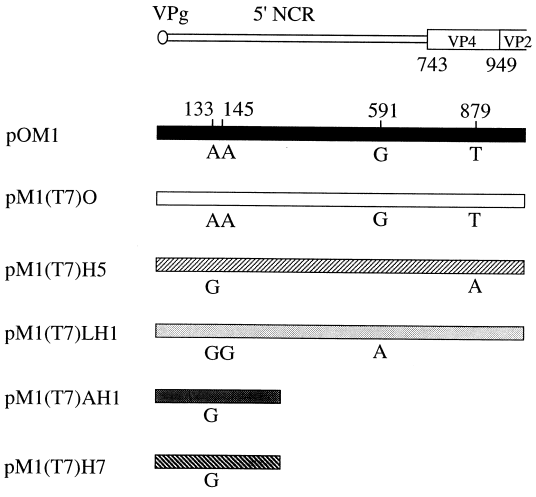

FIG. 3. Nucleotide sequences of the 5′-proximal parts of poliovirus RNAs. Nucleotide sequences of pOM1, pM1(T7)0, pM1(T7)H5, pM1(T7)LH1, pM1 (T7)AH1, and pM1(T7)H7 are shown. The numbers are the nucleotide positions from the 5′ end of the genome. Portions of nucleotide sequences determined are represented by bars. Only nucleotides different between WT virus and mutants are indicated. The structure of the poliovirus genome is shown at the top.

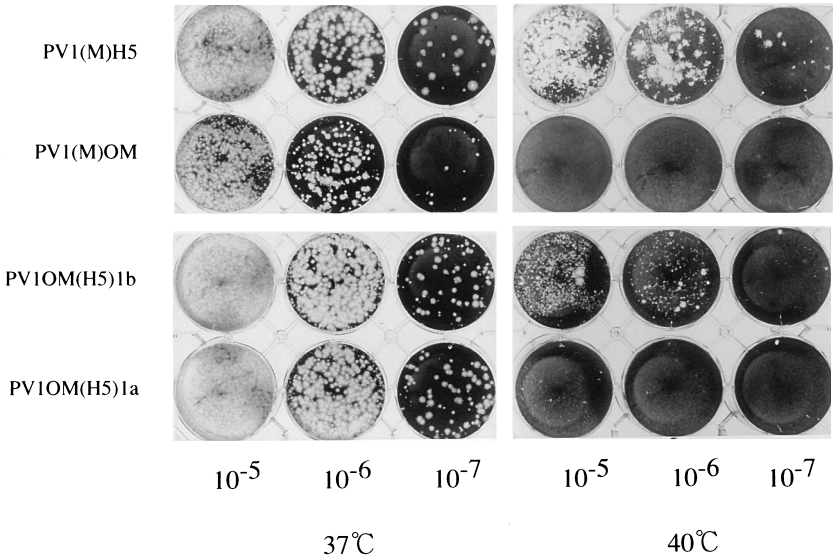

FIG. 4. Plaque forming ability of polioviruses. Lα cells were infected with diluted viruses of the WT Mahoney virus [PV1(M)OM], an H^r mutant [PV1(M)H5], and recombinants [PV1OM(H5)1b and PV1OM(H5)1α] and incubated under agar overlay at 37 or 40°C for 3 days. After fixation, the cells were stained with crystal violet.

nucleotide sequences of this region of the two Mahoney cDNAs [pOM1 and pM1(T7)0] were the same as previously published data (26). Two mutation sites at nt 133 (A to G) and 879 (T to A) were identified in pM1(T7)H5. Three mutation sites at nt 133 (A to G), 145 (A to G), and 591 (G to A) were identified in this region of pM1(T7)LH1. Of these, a mutation of nt 133 (A to G) was common to the two H^r mutant cDNAs. Accordingly, nucleotide sequence analysis of the genome region around nt 133 of the other two H^r mutants [PV1(M)AH1 and PV1(M)H7] was carried out. The result showed that nt 133 was also G in the genomes of those two H^r mutants (Fig. 3). It is therefore possible that a mutation at nt 133 from A to G within the 5′NCR gives the H^r phenotype to poliovirus type 1.

To determine the relationship between the nucleotide change at nt 133 (A to G) and the H^r growth phenotype, a single mutation (A to G) at nt 133 was introduced in the WT Mahoney genome by replacing the *Eco*RI and *Pin*AI fragment (nt 1 to 339) of Mahoney cDNA [pM1(T7)0 or pOM1] with the corresponding fragment of pM1(T7)H5 cDNA. The recombinant viruses recovered from pM1(H5)3b and pOM1(H5)1b (recombinants 12 and 14 in Fig. 2 and 4) showed the H^r growth phenotype. The reciprocal recombinants, pM1(H5)3a and pOM1(H5)1a (recombinants 11 and 13 in Fig. 2 and 4) showed the WT Mahoney phenotype. The results indicate that nt 133 is a key mutation site determining the H^r growth phenotype. Recombinants pM1(H5)2a (recombinant 9 in Fig. 2) and pM1(H5)2b (recombinant 10 in Fig. 2) showed the WT phenotype and H^r phenotype, respectively. This finding, together with the results described above, further confirmed that the H^r mutation site resided at G-133 and not in the region downstream of nt 340.

Contribution of G-133 to viral RNA synthesis. As shown in Fig. 1, synthesis of Mahoney virus RNA in Lα cells was restricted at 40°C, whereas that of H^r mutants proceeded to levels similar to those at 37°C. H^r mutants may overcome the restriction by a mutation at nt 133, since a single mutation at nt 133 (A to G) contributed to the H^r growth phenotype (Fig. 2 and 4). To investigate whether only one nucleotide change at nt 133 suppressed the restricted RNA synthesis of the WT Mahoney virus at 40°C in PVR-mouse cells, viral RNA synthesis in Lα cells of PV1OM(H5)1a and PV1OM(H5)1b (Table 1) was examined by [^{3}H]uridine incorporation into the trichloroacetic acid-insoluble fraction as described in Materials and Methods (Fig. 5). PV1OM(H5)1a and PV1OM(H5)1b are one-point mutants having A-133 in the H^r mutant PV1(M)H5 background and G-133 in the WT Mahoney virus PV1(M)OM background, respectively (Table 1; Fig. 2). [^{3}H]uridine incorporation is restricted at 40°C in Lα cells infected with PV1(M)OM and PV1OM(H5)1a but not with H^r mutant PV1(M)H5 or PV1OM(H5)1b (Fig. 5B). Viral RNAs were synthesized to similar levels at 37°C at 7 h postinfection for all these viruses

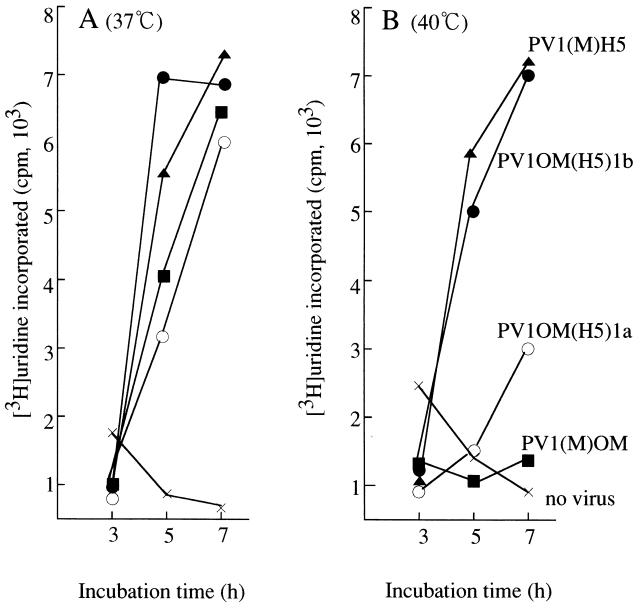

FIG. 5. RNA synthesis of recombinant polioviruses. Lα cells in 12-well plates were infected with the WT Mahoney virus [PV1(M)OM], an H^r mutant [PV1(M)H5], or recombinants [PV1OM(H5)1a and PV1OM(H5)1b] at an MOI of 20 and incubated at 37 or 40°C. Other conditions were the same as those described in the legend to Fig. 1.

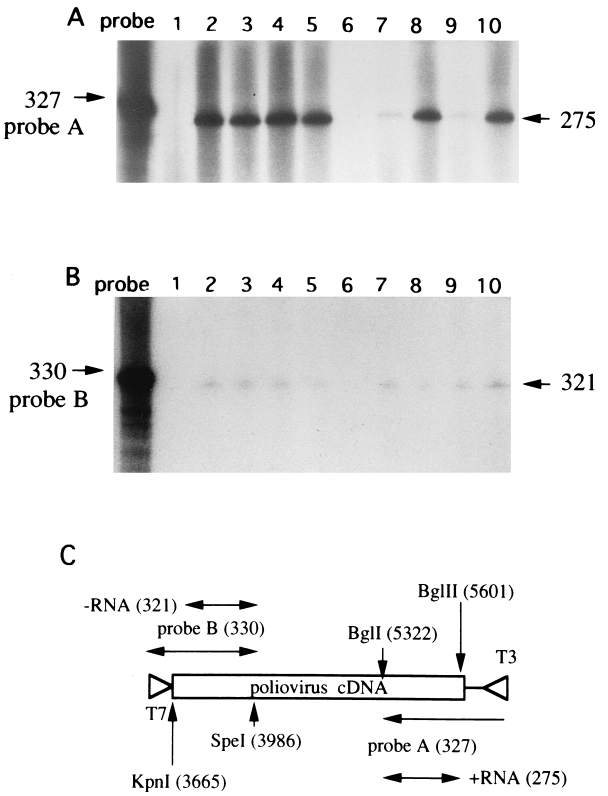

FIG. 6. RNase protection analysis. Lα cells were infected with no virus (mock infection; lanes 1 and 6), WT virus [PV1(M)OM] (lanes 2 and 7), an H^r mutant [PV1(M)H5] (lanes 5 and 10), or recombinants [PV1OM(H5)1b (lanes 3 and 8) and PV1OM(H5)1a (lanes 4 and 9)] at an MOI of 20 and incubated at 37°C (lanes 1 to 5) or 40°C (lanes 6 to 10). At 5 h postinfection, RNAs were extracted from the cells and hybridized with ^{32}P-labeled RNA probes as described in Materials and Methods. The RNase-resistant products were analyzed in 5% polyacrylamide gels in the presence of 8 M urea. Positive-strand (A) and negative-strand (B) RNAs were detected. Labeled RNA probes (5×10^5 cpm) alone were used in the probe lanes. Sizes are indicated in nucleotides.

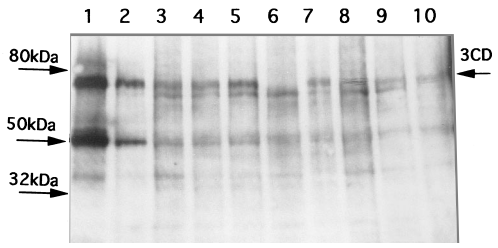

FIG. 7. Western blot analysis of viral proteins in Lα cells infected with poliovirus. Cells were mock infected (lane 6) or infected with WT Mahoney virus [PV1(M)OM] (lanes 1, 2, and 7), an H^r mutant [PV1(M)H5] (lanes 5 and 10), or recombinants [PV1OM(H5)1a (lanes 4 and 9) and PV1OM(H5)1b (lanes 3 and 8)] and grown at 37°C (lanes 1 to 6) or 40°C (lanes 7 to 10) in the presence (lanes 2 to 10) or absence (lane 1) of 1 mM guanidine hydrochloride. At 7 h postinfection, extracts were prepared and analyzed by Western blotting as described in Materials and Methods. The sizes of protein markers (prestained; Bio-Rad) are indicated on the left, and the position of 3CD (72 kDa) is indicated on the right.

in Lα cells (Fig. 5A). These results indicate that G-133 contributes to efficient synthesis of poliovirus RNA in infected Lα cells at 40°C.

As reported previously, synthesis of plus-strand RNA of Mahoney virus was restricted at 40°C in PVR-mouse cells (30). A one-point mutant containing G-133 in the WT Mahoney background [PV1OM(H5)1b] may overcome this restriction. Accordingly, an RNase protection assay was carried out with cytoplasmic RNAs from infected Lα cells at 37 or 40°C. As shown in Fig. 6, plus-strand RNAs of H^r mutants PV1(M)H5 and PV1OM(H5)1b synthesized at 40°C were detected at levels similar to those at 37°C. However, only low levels of plus-strand RNAs of the Mahoney virus and PV1OM(H5)1a were detected at 40°C, although considerable amounts of the RNAs were detected at 37°C. Levels of minus-strand RNAs of all the viruses used appear not to be significantly different from each other at either temperature. These results indicate that the restricted synthesis of Mahoney virus plus-strand RNA in Lα cells at 40°C is suppressed by a one-point mutation of A to G at nt 133.

Influence of G-133 on viral protein synthesis. According to a published scheme of secondary structure of the 5′NCR of poliovirus RNA (38), nt 133 is located in a stem-loop II structure which is considered to play an important role(s) in the function of the IRES (19, 22). Thus, the nucleotide change at nt 133 may influence the efficiency of viral protein synthesis. To examine translation of infected virion RNA, Lα cells were infected with WT Mahoney virus and H^r mutants in the presence of 1 mM guanidine hydrochloride. As shown in Fig. 1, the synthesis of poliovirus RNA was inhibited in the presence of 1 mM guanidine hydrochloride. A sensitive assay for poliovirus proteins translated early in the infection cycle is to monitor the inhibition of host cell protein synthesis (30). An experiment similar to one previously described (30) was carried out to detect poliovirus protein synthesis early in the infectious cycle. The results indicate that inhibition of cellular protein synthesis of H^r mutants appears not to be enhanced at 40°C (data not shown). Viral proteins in the infected cells were examined by Western blotting with anti-3D antibodies as described in Materials and Methods. The viral 3CD protein was detected in lysates of Lα cells infected with the WT Mahoney, H^r mutant, and recombinant viruses at 37 or 40°C (Fig. 7). Enhanced translation efficiencies of H^r mutants were not observed at 40°C. These results suggest that only a point mutation at G-133 in the stem-loop II structure (Fig. 8) does not affect translation in Lα cells at 37 or 40°C, although the results are not quantitatively reliable. A preliminary RNA transfection experiment involving recombinant dicistronic mRNAs, in which expression of the second cistron is controlled by IRES, supported these results (data not shown).

Requirement of G-133. All H^r mutants analyzed in this study contained G at nt 133. Since a strong inclination toward transition mutation was observed in the nucleotide substitutions of poliovirus RNA (16), it is possible that G-133 mutants are

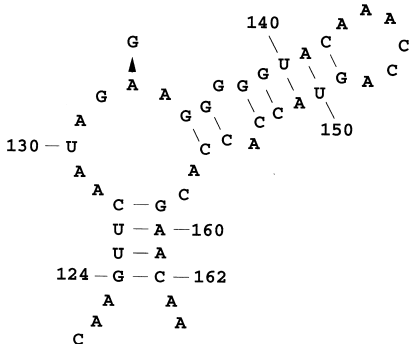

FIG. 8. Possible secondary structure of a stem-loop II structure (nt 124 to 162) in the 5′NCR of poliovirus type 1 Mahoney RNA.

TABLE 3. Sensitivity of cells to mutant poliovirus infection

Virus	Titer (PFU/ml)			
	Lα cells		HeLa cells	
	37°C	40°C	37°C	40°C
PV1(M)OM	2×10^8	$\leq 10^4$	2×10^8	10^8
PV1(M)OM(133G)[a]	10^8	3×10^7	2×10^8	10^8
PV1(M)OM(133C)	10^8	$\leq 10^4$	5×10^7	7×10^7
PV1(M)OM(133U)	4×10^7	$\leq 10^4$	1.5×10^8	10^8
PV1(M)OM(133del)	2×10^8	$\leq 10^4$	1.5×10^8	2×10^8
PV1(M)OM(134G)	10^8	$\leq 10^4$	2×10^8	1.5×10^8

[a] PV1OM(H5)1b.

preferentially isolated as H^r mutants and that mutations other than the transition at nt 133 also yield an H^r phenotype. To test this possibility, Mahoney viruses with a single mutation of C-133 or U-133 or a deletion mutation at nt 133 were prepared as described in Materials and Methods and designated PV1 (M)OM(133C), PV1(M)OM(133U), and PV1(M)OM(133del) respectively. Mahoney virus with a single mutation of G-134 [PV1(M)OM(134G)] was also prepared to determine if G-133 is required for the H^r phenotype. Growth phenotypes of these single mutants were examined in Lα and HeLa cells at 37 and 40°C (Table 3). All the viruses containing a single mutation at nt 133 except the G-133 mutant [PV1OM(H5)1b] showed the phenotype of WT Mahoney virus [PV1(M)OM]). The G-134 mutant was also demonstrated to have a WT phenotype. These results suggest that a point mutation of A to G at nt 133 is required for the H^r growth phenotype in Lα cells.

DISCUSSION

We have previously shown that viral growth and synthesis of plus-strand RNA of poliovirus type 1 (Mahoney strain) are restricted at 40°C in PVR-mouse cells, although growth of mouse cells was not affected at this temperature (30). To identify the genome region involved in the restricted growth phenotype, H^r growth mutants in PVR-mouse cells at 40°C were isolated and analyzed by molecular genetic methods. The key mutation of the H^r mutants was identified at nt 133 (A to G) within the 5′NCR region of the RNA. An H^r growth mutant with a single mutation of G-133 showed a normal level of plus-strand RNA synthesis at 40°C in PVR-mouse cells. If the viral translation efficiency of the H^r mutant appeared not to be enhanced at 40°C compared with that at 37°C, this observation strongly suggests that the restricted growth of the Mahoney virus at 40°C in PVR-mouse cells is mainly due to a lowered efficiency of the synthesis of viral plus-strand RNA.

All four H^r mutants contained G-133, which is a transition mutation. The frequency of isolation of an H^r mutant was approximately 10^{-4}, which is in agreement with the frequency of errors made by poliovirus replicase (16). Since transversions have been thought to be approximately 50-fold less frequent than transitions (16), it was possible that H^r mutants with a transversion mutation at nt 133 failed to be isolated. However, recombinant single mutants with a transversion mutation, C-133 or U-133, did not show an H^r growth phenotype (Table 3). Furthermore, a deletion at nt 133 or the G-134 mutation did not yield an H^r growth phenotype (Table 3). Thus, G, but not other nucleotides, at nt 133 is required for the H^r growth phenotype in Lα cells. The reason for this phenomenon is unclear at present. However, it is possible that interaction of this genome region with a mouse cellular factor(s) involved in viral plus-strand RNA synthesis is made H^r by the nucleotide change from A to G at nt 133. It is of interest that the MEF-1 strain of poliovirus type 2 and the Leon strain of poliovirus type 3 have G's at the corresponding nt 133 of poliovirus type 1 RNA (14b, 33) and show the H^r phenotype in Lα cells (data not shown). These observations suggest that G-133 is very important for the expression of the H^r phenotype of poliovirus, although mutations in other genome regions may also give an H^r phenotype to Mahoney virus.

The 5′NCR of poliovirus RNA contains signals important for virus-specific RNA replication and translation. These two functions are reported to be expressed by independent domains (28); that is, the 5′-terminal cloverleaf-like structure (nt 1 to 88) and the remaining structure (downstream of nt 120) are for viral RNA synthesis and viral translation, respectively. Indeed, the stem-loop II structure (nt 124 to 162) has been shown to be involved in IRES function (22, 31) (Fig. 8). Furthermore, Angel del Maria et al. (4) have reported that two segments, nt 97 to 182 and nt 510 to 629, are involved in interaction with eukaryotic elongation factor 2α. However, it is demonstrated here that the RNA region around nt 133 within the stem-loop II structure participates in plus-strand viral RNA synthesis in addition to the 5′-proximal cloverleaf structure. This work, therefore, reveals a new *cis*-element for RNA replication of poliovirus. Borman et al. (7) have also suggested that the RNA segment (around nt 343 to 500) within the IRES controls viral RNA synthesis. Since it is apparent that the G-133 mutant does not have an enhanced efficiency in viral translation at 40°C in PVR-mouse cells, this mutation at nt 133 may not affect the IRES function. Thus, the stem-loop II structure seems to have a dual function: RNA synthesis and translation of poliovirus. Precise mutational analyses are now being carried out in regard to structure and function of stem-loop II.

Andino et al. (1, 2) have suggested that a 36-kDa host cellular protein is required for 3CD binding to the 5′ cloverleaf-like structure of the viral plus-strand RNA and that formation of the RNP complex involving the cellular and viral proteins plays an important role(s) in the initiation of plus-strand RNA synthesis. The 36-kDa protein, however, might be a cleaved product of eukaryotic elongation factor EF-1α (50 kDa) (14). In vitro binding experiments have demonstrated that 3CD binding was observed without cellular factors in the presence of the purified viral polyprotein 3AB (14). Thus, a host cellular factor(s) required for poliovirus RNA replication has not yet been truly identified. It is conceivable, however, that a nucleotide change from A to G at nt 133 influences the interaction between the 5′NCR and a mouse cellular factor(s) involved in viral plus-strand RNA synthesis, resulting in expression of the H^r phenotype. Since a single mutation to G but not other mutations at nt 133 resulted in the H^r growth phenotype, it is possible that a putative mouse cellular factor(s) recognizes the primary structure around nt 133 or the corresponding region of the minus-strand RNA. Alternatively, the substitution at nt 133 of A by G may cause a more global change which affects protein binding at a site distant from nt 133. In any event, we are now trying to identify the host cellular factor(s) related to the expression of the H^r phenotype.

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