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In Vitro Phenotypic Markers of a Poliovirus Recombinant Constructed from Infectious cDNA Clones of the Neurovirulent Mahoney Strain and the Attenuated Sabin 1 Strain

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Infectious cDNA corresponding to the entire genome of the attenuated Sabin strain of type 1 poliovirus has been inserted into *Eco*RI site of bacterial plasmid pBR325. Two consecutive *Pst*I fragments (nucleotide positions 1814 to 3421) of the infectious cDNA of the Sabin 1 strain were replaced by the corresponding DNA fragments prepared from an infectious DNA clone of the genome of the virulent Mahoney strain of poliovirus type 1. The exchanged segment encodes capsid protein VP1 and part of capsid protein VP3, a region in which a large number of amino acid differences between the attenuated Sabin and the parental, neurovirulent Mahoney strain cluster. The recombinant virus was obtained by DNA transfection of HeLa S3 cells, and several in vitro phenotypes of the virus were compared with those of the parental viruses. The recombinant virus was recognized by a neutralizing monoclonal antibody specific to the Mahoney strain. Growth of the Sabin strain of poliovirus has been shown to be quite dependent upon the bicarbonate concentration (*d* marker). The growth of the recombinant virus, however, was not highly dependent upon the concentration of bicarbonate in cell culture media, and thus resembled that of the Mahoney strain. On the other hand, the temperature-sensitive multiplication (*rct* marker) and the small-plaque morphology of the recombinant virus corresponded to the phenotype of the Sabin 1 strain. The in vitro recombination of infectious cDNA clones of genomic RNA and subsequent analysis of the growth properties of the recombinant virus have allowed us to correlate specific mutations in the genome of an RNA virus with certain biological characteristics of that virus.

The genome of poliovirus, a picornavirus occurring in three serotypes, is a single-stranded RNA molecule of ca. 7,500 nucleotides that is 3′ polyadenylylated and linked at the 5′ end to a small protein called VPg (9 and references therein). This genomic RNA is of positive polarity and functions as mRNA after entry into the host cell cytoplasm. The RNA is translated into a single continuous polyprotein with a molecular weight of 246,000 (10). The polyprotein is subsequently cleaved by proteases to the viral structural and nonstructural proteins (6 and references therein).

Poliovirus is the causative agent of poliomyelitis. Attenuated strains of all three types of poliovirus have been isolated and used effectively as oral vaccines to control the paralytic disease (22). Apart from the differences in causing disease, virulent and attenuated poliovirus strains differ also in a number of biological characteristics. Some of these biological characteristics are utilized as in vitro marker tests (that is, tests carried out in tissue culture) to analyze the quality of a batch of oral live vaccines (12). These characteristics include plaque size, the sensitivity of growth at temperature above 37°C (*rct* marker), and the sensitivity to low concentration of bicarbonate in cell culture media (*d* marker). The different phenotypes of wild-type and attenuated virus strains are, of course, the results of differences in genome structures (mutations).

The Sabin vaccine strain of type 1 poliovirus was derived from the virulent Mahoney strain by multiple passage through host cells of nonhuman origin that resulted in multiple mutations leading to the attenuated phenotype (21, 23). The total nucleotide sequences of the genomes of both poliovirus strains are known (10, 16, 18). In the primary structure of the RNA of the two strains, 56 nucleotide differences were observed, and these nucleotide changes were scattered over the entire length of the genome (16, 27). Of the 56 nucleotide differences, 21 resulted in amino acid changes (16). Interestingly, a cluster of amino acid changes was located in the NH_2-terminal half of capsid protein VP1. Therefore, it appeared possible that mutations in the genome locus encoding VP1 contribute to some biological differences between the Mahoney and Sabin strains.

Racaniello and Baltimore (19) have shown that a complete, cloned cDNA copy of the genome of the virulent Mahoney strain of type 1 poliovirus is infectious in mammalian cells. A similar clone of high specific infectivity was also constructed by Semler et al. (24). Omata et al. (17a) isolated an infectious cDNA clone of the genome of the Sabin 1 vaccine strain and showed that it could be utilized as seed material for the oral polio live vaccine. The availability of infectious clones of poliovirus RNA provides a molecular genetic approach for investigating the relationship between

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genome structure and function of viral gene products using recombinant DNA technology. Thus, it is now possible to construct recombinant viruses between different strains, or even of different serotypes of polioviruses, and test the properties of the recombinants.

Here we report the construction of an infectious recombinant cDNA clone from segments of the virulent Mahoney and attenuated Sabin 1 genomes. Specifically, the Sabin 1 sequence that encodes predominantly VP1 has been replaced by the corresponding Mahoney-specific sequence. We also describe the biological properties of the recombinant poliovirus that was isolated from mammalian cells transfected with the corresponding DNA clone.

MATERIALS AND METHODS

Preparation of cDNA clones. Both full-length cDNAs of the genomes of the virulent Mahoney (24) and the attenuated Sabin 1 (17a) strains have been inserted into the *Eco*RI site of bacterial plasmid pBR325 by using *Eco*RI linkers. These cloned DNAs are infectious in mammalian cells and are designated in this report as pVM(1)pDS306 and pVS(1)IC-0(25), respectively.

In the course of constructing pVS(1)IC-0(25), a plasmid pB5EPPE which lacked two consecutive *Pst*I fragments (PP12; nucleotide positions 1814 to 3421; see Fig. 1) was obtained as previously described (17a). The corresponding *Pst*I fragment (PPsl) of the Mahoney cDNA clone was prepared by joining two Mahoney-specific *Pst*I fragments (PPs and PPl) which had been cloned separately into the *Pst*I site of bacterial plasmid pBR322 (A. Nomoto and E. Wimmer, unpublished data).

Plasmid pB5EPPE was partially cleaved with *Pst*I and ligated to the fragment PPsl to form a recombinant cDNA between the genomes of the Mahoney and Sabin 1 strains. The resulting plasmid was designated as pVSM(1)IC-1a(25).

Conditions for digestion with restriction endonucleases and for ligation with T4 DNA ligase were essentially the same as described previously (11, 16, 17).

Transfection with DNA. HeLa S3 cells were grown in Eagle minimum essential medium supplemented with 5% newborn calf serum in 6-cm plastic dishes. The medium was removed when the cells had grown to ca. 70% confluency and replaced with 1 ml of Dulbecco modified Eagle medium containing 5% newborn calf serum. After the cultures were kept for 30 min at 36°C, 10 μg of DNA per plate was added as a calcium phosphate precipitate in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (5, 17a). The cells were kept for 2 h at 36°C and then washed twice with Eagle minimum essential medium and covered with the same medium containing 5% newborn calf serum. The cultures were incubated at 34°C for 5 days or at 37°C for 4 days.

PFU of the recovered virus in culture fluid were measured by using HeLa S3 cells as previously reported (2, 17a).

Virus multiplication. HeLa S3 monolayer cells were infected with viruses recovered from DNA-transfected cells at a multiplicity of infection of ca. 10^{-3} (17a). After an incubation at 34°C for 5 days or at 37°C for 4 days, the viruses were obtained by freeze-thawing three times, followed by centrifugation to remove cell debris, and used for biological characterizations. The viruses were further passaged once in suspension-cultured HeLa S3 cells to make high-titer stocks as previously described (17a). These virus stocks were used as inocula for the structural analyses of RNAs.

For isolation of ^{32}P-labeled virus, virus-infected HeLa S3 cells were incubated in phosphate-free media in the presence of ^{32}P at a concentration of 20 μCi/ml as previously reported (14). ^{32}P-labeled viral RNAs were prepared by phenol-chloroform extraction from purified ^{32}P-labeled virions and purified by sucrose density gradient centrifugation as previously described (14).

Enzyme-linked immunosorbent assay. Neutralizing monoclonal antibodies specific to the Mahoney strain and the Sabin 1 strain were generous gifts of Mineo Arita, National Institute of Health of Japan. Antigenicities of viruses were tested by the modified method of enzyme-linked immunosorbent assay (28).

A microELISA plate (immulon M-129A; Dynatech Laboratories) was coated with 50 μl of the strain-specific monoclonal antibody (10^{-4} dilution with 0.05 M sodium carbonate [pH 9.6]) at 4°C overnight. After the plate was rinsed with 200 μl of washing buffer containing phosphate-buffered saline and 0.05% Tween 20 three times for 3 min each, 50 μl of a virus solution (ca. 3×10^7 PFU/ml in dilution buffer containing 0.01 M phosphate-buffered saline, 0.5% Tween 20, and 1% bovine serum albumin) were added. The plate was kept at 37°C for 1 h and washed five times (for 3 min each) with 250 μl of washing buffer. An amount of 50 μl of guinea pig anti-type 1 poliovirus serum (10^{-4} dilution in a dilution buffer) was added, and the plate was kept at 37°C for 1 h. The plate was then washed five times as above, incubated at 37°C for 1 h with 50 μl of 1-μg/ml biotinylated goat anti-guinea pig immunoglobulin G (Vector Laboratories) in dilution buffer, and washed again with the washing buffer. The plate was then incubated with 50 μl of Avidin D (1 μg/ml) conjugated with horseradish peroxidase (Vector) in dilution buffer, kept at 15°C for 10 min, and washed five times as above. The enzyme reaction was carried out at 37°C for 10 min in the dark with 100 μl of a freshly prepared enzyme substrate solution (pH 5.0) containing 0.04% orthophenylene diamine, 0.006% H_2O_2, 0.05 M citric acid, and 0.1 M dibasic sodium phosphate, and stopped by addition of 50 μl of 2 M H_2SO_4. The A_{492} of the reaction mixture was measured in each well.

In vitro phenotypic marker tests. The *rct* (reproductive capacity at different temperatures) marker test was carried out by using primary-cultured cynomolgus monkey kidney cells at temperatures of 36, 39, 39.5, and 40°C as described previously (17a). After an incubation of 7 days, plaques were counted. Similarly, the *d* (delayed) marker test was performed at 36°C at various concentrations of sodium bicarbonate, that is 0.225, 0.08, and 0.03% as previously reported (17a). Plaques were counted after an incubation for 4 days.

To analyze virus plaque morphologies, monolayers of line African green monkey kidney cells were infected with viruses. After incubation for 3, 4, 5, or 7 days at 36°C under agar overlays, the cells were stained with crystal violet, and the diameters of plaques were measured.

Analysis of DNA and RNA. Restriction fragments of DNA were analyzed and purified in 0.8 to 1.0% agarose gels in a buffer containing 40 mM Tris base, 2 mM EDTA, and 20 mM sodium acetate (pH adjusted to 8.0 with acetic acid). *Hin*dIII digests of phage λ DNA were used as molecular weight standards.

Fingerprint analysis of RNase T1 digests of ^{32}P-labeled virion RNAs were carried out by two-dimensional polyacrylamide gel electrophoresis as previously described (14, 15).

RESULTS

Construction of an infectious recombinant DNA clone of poliovirus. The total nucleotide sequences of the genomes of

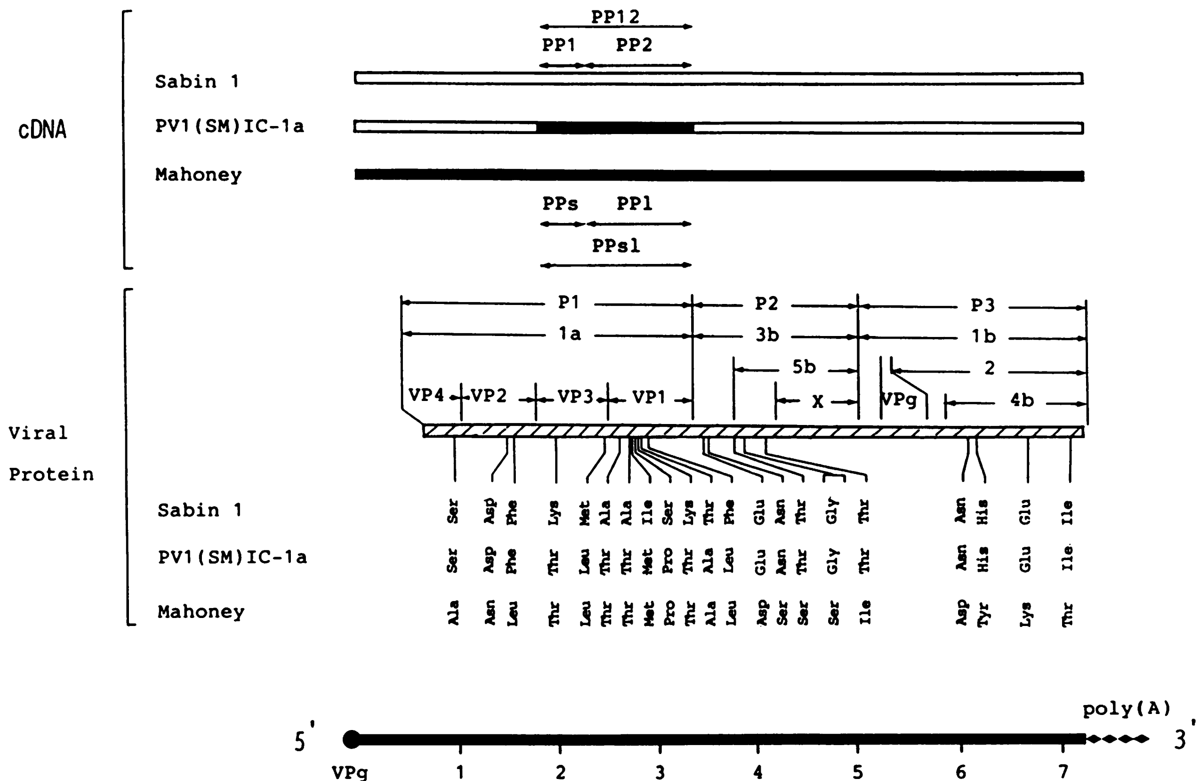

FIG. 1. Construction of the recombinant type 1 poliovirus genome and locations of amino acid differences in polypeptides of the Mahoney and Sabin 1 strains. Open and closed bars represent the nucleotide sequences of cDNAs specific to Sabin 1 and Mahoney strains, respectively. PP1, PP2, and PP12 are *Pst*I fragments of Sabin 1-specific cDNA. PPs, PPl, and PPsl are *Pst*I fragments of Mahoney-specific cDNA. The hatched bar represents the viral polyprotein (NCVP00). The protein map is indicated above the bar. P1, P2, and P3 represent the protein region for coat proteins, X proteins, and replication proteins, respectively. The locations of amino acid differences between Mahoney and Sabin 1 strains are indicated by lines under the hatched bar, as are the amino acids specific to Mahoney and Sabin 1 strains. Genome RNA (marked in kilobases from the 5′ terminus) is shown at the bottom of the figure. Poly(A), Polyadenylate.

the virulent Mahoney and the corresponding attenuated Sabin strains of type 1 poliovirus have been determined (10, 16, 18). When the predicted amino acid sequences of the viral polypeptides were compared, a cluster of amino acid changes was observed in the NH_2-terminal half of capsid protein VP1 (16) (Fig. 1). Since VP1 is not only the largest of the capsid proteins but also the most exposed surface protein of the virion (31), we initiated "allele replacement" experiments to determine how these amino acid changes might influence the biological properties of the virus.

The isolation of clones containing full-length cDNA copies of the genomes of the poliovirus Mahoney and Sabin 1 strains inserted into the *Eco*RI site of bacterial plasmid pBR325 has been described (17a, 24). In this report the plasmids were designated as pVM(1)pDS306(25) and pVS(1)IC-0(25) for the Mahoney and Sabin derivatives, respectively. Both plasmids are infectious in mammalian cells. To analyze the effect of amino acid changes, clustered predominantly in VP1, on the phenotype of the virus, we constructed a recombinant cDNA clone, in which a PP12 fragment (nucleotide positions 1814 to 3421) of an infectious cDNA clone of Sabin 1 strain was replaced by the corresponding fragment (PPsl) of Mahoney strain as described above (Fig. 1). The newly constructed plasmid, named pVSM(1)IC-1a(25), was infectious (data not shown).

Analysis of viral RNA. HeLa S3 cells were transfected with closed circular forms of pVM(1)pDS306(25), pVS(1)IC-0(25), and pVSM(1)IC-1a(25), and the corresponding viruses, designated as PV1(M)pDS306, PV1(Sab)IC-0, and PV1(SM)IC-1a, respectively, were recovered from the cells as described above. Since the PV1(SM)IC-1a, like the PV1(Sab)IC-0, showed temperature sensitivity in its multiplication (see below), this isolate was grown at 33.5 to 34°C, whereas PV1(M)pDS306 was grown at 37°C.

Based on mapping experiments (13) and sequence studies (10, 15), the precise location of large RNase T1- and RNase A-resistant oligonucleotides within the Mahoney and Sabin 1 genomes and their position in fingerprints have been established (15). Accordingly, a fingerprint of an RNase T1 digest of PV1(SM)IC-1a RNA should show a Mahoney-specific spot (oligonucleotide 30) in addition to all the Sabin 1-specific spots (15). We, therefore, analyzed the genome RNA of PV1(SM)IC-1a by fingerprinting after RNase T1 digestion and compared the pattern with those obtained from genomes of PV1(M)pDS306 and PV1(Sab)IC-0. As shown in Fig. 2, the fingerprints obtained from PV1(M)pDS306 and PV1(Sab)IC-0 genomes were identical to previously reported patterns of the Mahoney and Sabin 1 genomes (15), whereas the fingerprint of PV1(SM)IC-1a genome RNA (Fig. 2B) showed the expected Mahoney-specific spot (oligonucleotide 30) and the Sabin 1-specific spots, oligonucleotide 37 (LSc), oligonucleotide a(LSc), and oligonucleotide 9(LSc). This result is compatible with the predicted genome structure of the recombinant virus PV1(SM)IC-1a.

Antigenicity. The segment exchanged in the recombinant virus codes for capsid protein VP1 and a portion of capsid

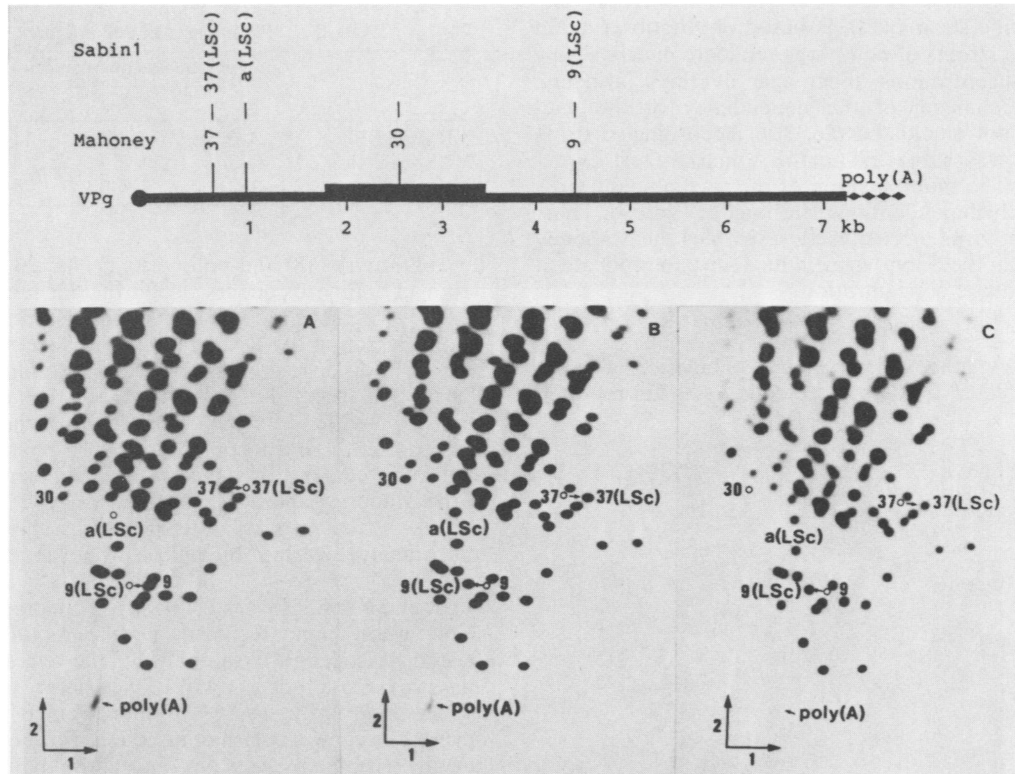

FIG. 2. Fingerprint analysis of genomes of the recombinant virus and its parent viruses. The genome structure of poliovirus (Fig. 1) has a closed bar to indicate the exchanged genome region. Established map positions of spots in fingerprint analyses that differ between the Mahoney and Sabin 1 strains are indicated by lines above the bar (see reference 15). The spot numbers specific to Mahoney and Sabin 1 strains are also shown. Fingerprints of RNase T1-resistant oligonucleotides of PV1(M)pDS306 (A), PV1(SM)IC-1a (B), and PV1(Sab)IC-0 (C) were obtained by the procedure described in the text. The nomenclature of the spots is as reported by Nomoto et al. (15). Open circles represent spots missing in (A) or (C) as compared with (C) or (A), respectively. Shifts of spots due to pyrimidine transition are indicated by arrows.

protein VP3. All neutralizing monoclonal antibodies isolated so far for poliovirus type 1 have been found to recognize only those neutralization epitopes that map in VP1 (3, 4, 29, 32). It is therefore possible to use this property to detect intra-typic differences of Mahoney and Sabin 1 strains. Monoclonal antibodies specific to the Mahoney and the Sabin 1 strains were employed to test the antigenicity of PV1(SM)IC-1a by an enzyme-linked immunosorbent assay procedure as described above. The recombinant virus was recognized only by a neutralizing monoclonal antibody specific to the Mahoney strain (Table 1). This result clearly indicated that the intra-typic antigenic determinant mapped in the genome locus represented by segment PPsl (Fig. 1).

Plaque size. Plaque size is one of the in vitro markers indicative of the multiplication level of viruses, and it might correlate with viral virulence (21). Indeed, the plaque size of the Mahoney strain is larger than that of the Sabin 1 strain. It was, therefore, of interest to measure the size of plaques produced by PV1(SM)IC-1a and to compare that with those

TABLE 1. Recognition of recombinant viruses by monoclonal antibodies

Virus	Antibody (A_{492})	
	Anti-PV1(Sab)	Anti-PV1(M)
PV1(M)pDS306	0.000	0.380
PV1(SM)IC-1a	0.000	0.373
PV1(Sab)IC-0	0.407	0.081

of PV1(M)pDS306 and PV1(Sab)IC-0. African green monkey kidney cells were infected with PV1(M)pDS306, PV1(Sab)IC-0, and PV1(SM)IC-1a and cultured as described above. Plaques as they appeared on the third, fifth, and seventh days are shown in Fig. 3, and the diameters of plaques on the third, fourth, fifth, and seventh days are given in Table 2. The plaque size of PV1(SM)IC-1a is smaller than that of PV1(M)pDS306 and is almost equal to that of the vaccine strain PV1(Sab)IC-0 (Fig. 3 and Table 2), although the recombinant virus was recognized by Mahoney-specific monoclonal antibody. It is likely that the decreased plaque size is the consequence of a decrease in the level of multiplication of the recombinant virus.

Sensitivity to temperature. The *rct* marker test is based on the temperature sensitivity of multiplication of the poliovirus vaccine strains (12), and it is one of the most reliable tests among many in vitro biological marker tests to analyze the quality of a poliovirus vaccine strain. Accordingly, the *rct* marker test was performed on PV1(SM)IC-1a as described above (Table 3). The recombinant virus was quite temperature sensitive when compared with PV1(M)pDS306, although the sensitivity is a little less than that of PV1(Sab)IC-0 (Table 3). These results indicate that mutations in the replaced genome region do not significantly contribute to the temperature sensitivity of the Sabin 1 strain. It also suggests that the Sabin 1 strain is a multistep temperature-sensitive mutant.

Dependency on bicarbonate concentration. The *d* (delayed) marker test, frequently used as an in vitro test to evaluate

poliovirus vaccine strains (12), is based on the observation that the vaccine strains of poliovirus replicate poorly at low bicarbonate concentrations under agar overlays, although the precise mechanism of the dependency of the vaccine strains is not elucidated (26, 30). Accordingly, strain PV1(SM)IC-1a was subjected to the *d* marker test as described above. The multiplication of the recombinant virus was only partially dependent on bicarbonate (Table 4). Thus, the recombinant virus more closely resembles the Mahoney strain rather than the Sabin virus in its ability to replicate at reduced bicarbonate concentration.

DISCUSSION

Biochemical evidence for genetic recombination of picornaviruses in infected tissue culture cells has been reported

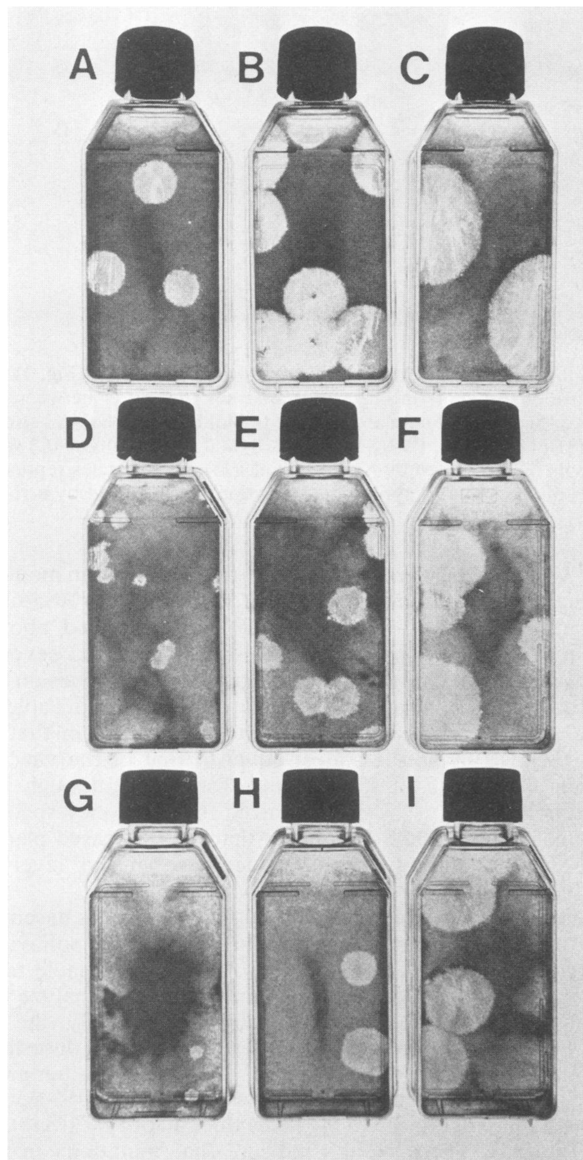

FIG. 3. Plaques displayed on a given day after inoculation of type 1 polioviruses. Strains PV1(M)pDS306 (A, B, and C), PV1(SM)IC-1a (D, E, and F), and PV1(Sab)IC-0 (G, H, and I) were inoculated as described in the text. After 3 (A, D, and G), 5 (B, E, and H), and 7 (C, F, and I) days of incubation at 36°C, the cells were stained with crystal violet.

TABLE 2. Plaque size of recombinant viruses

Virus	Plaque size (mm) on day after inoculation:			
	3	4	5	7
PV1(M)pDS306	4–8	6–10	11–12	15–18
PV1(SM)IC-1a	1–2	4–7	4–10	8–15
PV1(Sab)IC-0	1–2	3–6	5–9	8–13

for aphthovirus (8) and poliovirus (1, 4a, 20, 25). Recombination of poliovirus occurs also in vaccine recipients (7). The crossover in these recombinants appears to be located in the central region of the RNA genome. Failure to select recombinants in other regions may be due to limiting numbers of selectible markers. Recombinant DNA technology will now make it possible to exchange any locus of the viral genome. We reported here the construction of a poliovirus recombinant from segments of cloned cDNA representing sequences of the Mahoney and Sabin 1 strains of type 1 poliovirus. In vitro tests, that is, tests in tissue culture, have been carried out to analyze certain biological properties of the recombinant.

The recombinant virus PV1(SM)IC-1a appeared to be less stable when compared with both parental strains. After several passages in tissue culture, the average plaque size increased (data not shown). Since large plaque variants appeared abruptly, these variants seemed to be selected during subsequent cycles of infection. Moreover, faint spots became visible in the RNA fingerprint patterns (data not shown), an observation indicating that the primary structure of the recombinant genome was undergoing sequence variation. Such variation is not seen with the parental strains, even after multiple passages in the same cells. Replacement of one part of a viral coat protein by elements of the coat protein region of other strains might cause alterations in the tertiary structure of the virion particle, leading to problems in RNA packaging or in particle stability. Variants with more stable virion structure might therefore be selected during multiple rounds of virus replication.

The genome segment used to exchange elements of type 1 poliovirus strains codes for capsid polypeptides that show a high degree of amino acid variation between the three serotypes of poliovirus (27). The high degree of amino acid variation in this region among serotypes must have evolved without altering capsid stability or the correct folding required for the faithful processing of the precursor proteins. Whether capsid regions of type 2 or type 3 poliovirus can be exchanged with those of type 1 is not presently known.

The replacement of the Sabin 1-specific sequence (positions 1814 to 3421) by the corresponding Mahoney-specific sequence resulted in a change of the intra-typic antigenicity of the virus. Plaque size and temperature-sensitivity (*rct* marker) of the recombinant virus, however, correlated to the phenotype of the Sabin 1 strain. The bicarbonate concentration dependency (*d* marker), on the other hand, corre-

TABLE 3. Reproductive capacity at different temperatures ($\log_{10}$ $PFU \cdot ml^{-1}$)

Virus	PFU at indicated temp (°C):				Log difference between temps (°C):		
	36	39	39.5	40	36/39	36/39.5	36/40
PV1(M)pDS306	7.90			6.87			1.03
PV1(SM)IC-1a	7.31	3.92	2.27	<0.39	3.39	5.04	>6.92
PV1(Sab)IC-0	7.43	2.53	<0.39		4.90	>7.04	

TABLE 4. Delayed marker test of viruses ($\log_{10}$ PFU·ml^{-1})

Virus	PFU at indicated sodium bicarbonate concn (%):			Log difference between concns (%):	
	0.03	0.08	0.225	0.225/0.03	0.225/0.08
PV1(M)pDS306	7.63	7.93	7.81	0.18	−0.12
PV1(SM)IC-1a	6.01	7.12	7.09	1.08	−0.03
PV1(Sab)IC-0	3.40	6.15	7.24	3.84	1.09

sponded more to the virulent Mahoney strain. Although the phenotype of the recombinant is influenced greatly by the allele replacement, the relationship between the folding of VP1 and VP3 and the corresponding phenotype remains unknown. A more precise mapping of these in vitro phenotypic markers is currently being carried out.

Since the very recent report by Agol et al. (1) has suggested that major determinants of neurovirulence reside in the capsid half of the poliovirus genome, and since the biological tests used in this study are the same as those used to evaluate the oral polio live vaccines, it will be of particular interest to know whether the recombinant virus is virulent or avirulent. Quantitative neurovirulence tests in experimental animals (monkeys) by many routes of inoculation will be necessary to answer this question. Very preliminary results have indicated that the virus was not fully virulent (unpublished data).

Although the data presented in this paper describe only a relatively simple exchange of genetic elements of two viral strains, the strategy employed here will allow us to define those characteristics of the vaccine strain that contribute to the attenuated phenotype of poliovirus type 1. This, in turn, will be of great interest if more stable vaccine strains of the type 2 and type 3 polioviruses are to be constructed. Knowledge of the molecular basis of many biological differences between virulent and attenuated poliovirus strains should also provide useful information for evaluating polio live vaccines by in vitro marker tests. Finally, allele replacement between different strains or even between different types or species of picornaviruses in vitro will be a useful tool in deciphering the molecular biology of these viruses.

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LITERATURE CITED

1. **Agol, V. I., V. P. Grachev, S. G. Drozdov, M. S. Kolesnikova, V. G. Kozlov, N. M. Ralph, L. I. Romanova, E. A. Tolskaya, A. V. Tyufanov, and E. G. Viktorova.** 1984. Construction and properties of intertypic poliovirus recombinants: first approximation mapping of the major determinants of neurovirulence. Virology **136**:41–55.
2. **Detjen, B. M., J. Lucas, and E. Wimmer.** 1978. Poliovirus single-stranded RNA and double-stranded RNA: differential infectivity in enucleate cells. J. Virol. **27**:582–586.
3. **Emini, E. A., B. A. Jameson, A. J. Lewis, G. R. Larsen, and E. Wimmer.** 1982. Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. J. Virol. **43**:997–1005.
4. **Emini, E. A., B. A. Jameson, and E. Wimmer.** 1984. The identification of multiple neutralization antigenic sites on poliovirus type 1 and the priming of the immune response with synthetic peptides, p. 65–75. *In* R. Chanock and R. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

4a. **Emini, E. A., J. Leibowitz, D. C. Diamond, J. Bonin, and E. Wimmer.** 1984. Recombinants of Mahoney and Sabin strain poliovirus type 1: analysis of *in vitro* phenotypic markers and evidence that resistance to guanidine maps in the non-structural proteins. Virology **137**:74–85.

5. **Graham, F. L., A. J. Van der Eb, and H. L. Heijneker.** 1974. Size and location of the transforming region in human adenovirus type 5 DNA. Nature (London) **251**:687–691.
6. **Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. U.S.A. **79**:3973–3977.
7. **Kew, O. M., and B. K. Nottay.** 1984. Evolution of the oral polio vaccine strains in humans occurs by both mutation and intermolecular recombination, p. 357–362. *In* R. Chanock and R. Lerner (ed.), Modern approaches to vaccines. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **King, A. M. Q., D. McCahon, W. R. Slade, and J. W. I. Newman.** 1982. Recombination in RNA. Cell **29**:921–928.
9. **Kitamura, N., C. Adler, and E. Wimmer.** 1980. Structure and expression of the picornavirus genome. Ann. N.Y. Acad. Sci. **345**:183–201.
10. **Kitamura, N., B. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
11. **Kuge, S., O. Chisaka, M. Kohara, N. Imura, K. Matsubara, and A. Nomoto.** 1984. Expression of poliovirus complementary DNA coding for viral antigenic determinants in *Escherichia coli*. J. Biochem. **96**:305–312.
12. **Nakano, J. H., M. H. Hatch, M. L. Thieme, and B. Nottay.** 1978. Parameters for differentiating vaccine-derived and wild poliovirus strains. Prog. Med. Virol. **24**:178–206.
13. **Nomoto, A., A. Jacobson, Y. F. Lee, J. J. Dunn, and E. Wimmer.** 1979. Defective interfering particles of poliovirus: mapping of the deletion and evidence that the deletion in genome of DI(1), (2) and (3) is located in the same region. J. Mol. Biol. **128**:179–196.
14. **Nomoto, A., S. Kajigaya, K. Suzuki, and N. Imura.** 1979. Possible point mutation sites in LSc,2ab poliovirus RNA and a protein covalently linked to the 5′-terminus. J. Gen. Virol. **45**:107–117.
15. **Nomoto, A., N. Kitamura, J. J. Lee, P. G. Rothberg, N. Imura, and E. Wimmer.** 1981. Identification of point mutations in the genomes of the poliovirus Sabin vaccine LSc,2ab, and catalogue of RNase T1- and RNase A-resistant oligonucleotides of poliovirus type 1 (Mahoney) RNA. Virology **112**:217–227.
16. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. U.S.A. **79**:5793–5797.
17. **Nomoto, A., H. Toyoda, N. Imura, S. Noguchi, and T. Sekiya.** 1982. Restriction map of double-stranded DNA copy synthesized from poliovirus Sabin 1 RNA. J. Biochem. **91**:1593–1600.

17a. **Omata, T., M. Kohara, Y. Sakai, A. Kameda, N. Imura, and A. Nomoto.** 1984. Cloned infectious complementary DNA of poliovirus Sabin 1 genome: biochemical and biological properties of recovered virus. Gene **32**:1–10.

18. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. U.S.A. **78**:4887–4891.
19. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
20. **Romanova, L. I., E. A. Tolskaya, M. S. Kolesnikova, and V. I. Agol.** 1980. Biochemical evidence for intertypic recombination of poliovirus. FEBS Lett. **118**:109–112.

21. **Sabin, A. B.** 1957. Properties of attenuated polioviruses and their behavior in human beings. Spec. Publ. N.Y. Acad. Sci. **5**:113–127.
22. **Sabin, A. B.** 1984. Strategies for elimination of poliomyelitis in different parts of the world with use of oral polio vaccine. Rev. Infect. Dis. **6**(Suppl. 2):391–396.
23. **Sabin, A. B., and L. R. Boulger.** 1973. History of Sabin attenuated poliovirus oral live vaccine strains. J. Biol. Stand. **1**:115–118.
24. **Semler, B. L., A. J. Dorner, and E. Wimmer.** 1984. Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids Res. **12**:5123–5141.
25. **Tolskaya, E. A., L. I. Romanova, M. S. Kolesnikova, and V. I. Agol.** 1983. Intertypic recombination in poliovirus: genetic and biochemical studies. Virology **124**:121–132.
26. **Totsuka, A., K. Ohtaki, and I. Tagaya.** 1978. Aggregation of enterovirus small plaque variants and polioviruses under low ionic strength conditions. J. Gen. Virol. **38**:519–533.
27. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences for all three poliovirus serotype genomes: implication for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561–585.
28. **van der Marel, P., A. G. Hazendonk, and A. L. van Wezel.** 1981. D-antigen determination in polio vaccine production: comparison of gel diffusion and ELISA methods. Dev. Biol. Stand. **47**:101–108.
29. **van der Werf, S., C. Wychowski, P. Bruneau, B. Blondel, R. Crainic, F. Horodniceanu, and M. Girard.** 1983. Localization of a poliovirus type 1 neutralization epitope in viral capsid polypeptide VP1. Proc. Natl. Acad. Sci. U.S.A. **80**:5080–5084.
30. **Wallis, C., and J. L. Melnick.** 1968. Mechanism of plaque inhibition of poliovirus possessing the *d* marker. J. Gen. Virol. **3**:349–357.
31. **Wetz, K., and K. O. Habermehl.** 1979. Topographical studies on poliovirus capsid proteins by chemical modification and cross-linking with bifunctional reagents. J. Gen. Virol. **44**:525–534.
32. **Wimmer, E., B. A. Jameson, and E. A. Emini.** 1984. Poliovirus antigenic sites and vaccines. Nature (London) **308**:19.