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Genetic Analysis of the Attenuation Phenotype of Poliovirus Type 1

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Seven different recombinant viruses from the virulent Mahoney and the attenuated Sabin parental strains of type 1 poliovirus were constructed in vitro by using infectious cDNA clones. Monkey neurovirulence tests (lesion score, spread value, and incidence of paralysis) using these recombinant viruses revealed that the loci influencing attenuation were spread over several areas of the viral genome, including the 5′ noncoding region. In vitro phenotypic marker tests corresponding to temperature sensitivity of growth (*rct* marker), plaque size, and dependency of growth on bicarbonate concentration (*d* marker) were performed to identify the genomic loci of these determinants and to investigate their correlation with attenuation. Determinants of temperature sensitivity mapped to many areas of the viral genome and expressed strong but not perfect correlation with attenuation. Recombinant viruses with Sabin-derived capsid proteins showed a small-plaque phenotype, and their growth was strongly dependent on bicarbonate concentration, suggesting that these determinants map to the genomic region encoding the viral capsid proteins. Plaque size and the *d* marker, however, were found to be poor indicators of attenuation. Moreover, virion surface characteristics such as immunogenicity and antigenicity had little or no correlation with neurovirulence. Nevertheless, viruses carrying Sabin-derived capsid proteins had an apparent tendency to exhibit less neurovirulence in tests on monkeys compared with recombinants carrying Mahoney-derived capsid proteins. Our results suggest that the extent of viral multiplication in the central nervous system of the test animals might be one of the most important factors determining neurovirulence. Moreover, we conclude that the expression of the attenuated phenotype of the Sabin 1 strain of poliovirus is the result of several different biological characteristics. Finally, none of the in vitro phenotypic markers alone can serve as a good indicator of neurovirulence or attenuation.

Poliovirus, known since 1908 to be the causative agent of poliomyelitis (29), is a human enterovirus that belongs to one of the largest single families of human pathogens, the *Picornaviridae*. The virus, a nonenveloped ("naked") particle containing a single-stranded RNA genome of plus-strand polarity, occurs in three serotypes. Although humans are its only natural host, poliovirus can be transferred to monkeys, in which it also causes paralytic disease (29).

Two strategies have been pursued to control poliomyelitis (29). The first strategy involved the preparation of inactivated virus as a vaccine (35). The second strategy involved the isolation of attenuated virus strains, of which those of A. B. Sabin were found to be most effective as oral live vaccines (19, 34). The virtue of the oral polio vaccines has been widely recognized (for a review, see reference 19). The Sabin vaccine strains, however, similar to other live viral vaccines, have an inherent problem, which is the risk, albeit low, of the reversion from the attenuated to the neurovirulent phenotype upon passage through the vaccine recipient. Indeed, a very small number of cases of paralytic poliomyelitis continues to occur in countries with extensive oral polio vaccine programs (19). Experimental evidence strongly suggests that most of these cases are vaccine related (15, 20).

The attenuated Sabin 1 strain was derived from the virulent Mahoney strain of type 1 poliovirus by multiple passages through host cells of nonhuman origin (33, 34). In addition to their different potentials for causing disease, these two strains of virus differ in a number of biological characteristics. Some of these biological characteristics are used as in vitro marker tests to analyze the quality of batches of oral live vaccines (21, 45). These include the sensitivity of viral multiplication to elevated temperatures (*rct* [reproductive capacity at different temperatures] marker), the size of plaques produced in infected monolayers of primate cells, and the sensitivity of viral plaque-forming ability to low concentrations of bicarbonate under agar overlay (*d* [delayed] marker). Although much information has been gathered concerning the molecular biology of poliovirus, little is known about the molecular basis of attenuation. Similarly, the molecular events that influence the in vitro phenotypes of the Sabin strains have not been elucidated. Our recent efforts to solve this problem are the focus of this study.

Different biological characteristics of the Mahoney and

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Sabin 1 strains must be due to the differences in genome structures that resulted from the attenuation process used to create the Sabin 1 virus. Indeed, the elucidation of the total nucleotide sequence of the RNA genomes of both strains (16, 24, 30) has revealed 55 nucleotide substitutions (24, 40; see also Fig. 1) within the 7,441 total heteropolymeric bases. These nucleotide changes were found to be scattered over the entire length of the genome and result in 21 amino acid replacements within the viral polyprotein (24, 40; Fig. 1).

Genetic recombinants between the neurovirulent and attenuated poliovirus strains were isolated recently from infected tissue culture cells (2, 11). The genetic crossover in these recombinants was found to be located in the central region (P2 region) of the RNA genome (2, 11). Using such recombinants in tests of monkey neurovirulence, Agol et al. (1, 2) suggested that major determinants of neurovirulence reside in the 5'-terminal half of the poliovirus genome. The failure to isolate recombinants from infected cells with crossovers in regions other than in P2, probably because of the lack of suitable selectable markers, does not allow a more precise identification of genomic sequences involved in attenuation.

We recently constructed an infectious recombinant plasmid clone from cDNA segments of Mahoney and Sabin 1 RNA and used it to analyze the influence of the region coding predominantly for capsid protein VP1 on the biological properties of poliovirus type 1 (17). The in vitro construction of recombinants from the Mahoney and Sabin 1 parental strains of type 1 poliovirus was made possible because we and others have previously isolated highly infectious cDNA clones of the corresponding RNA genomes (28, 31, 36). Viruses isolated from HeLa cells transfected with either Mahoney or Sabin 1 cDNA clones were found to be indistinguishable from the parental Mahoney virus (31, 36) or from the parental attenuated Sabin 1 virus (28), respectively.

Here we report the results of monkey neurovirulence tests of recombinant viruses of the Mahoney and Sabin 1 strains constructed in vitro. Neurovirulence of these recombinant viruses will be correlated with in vitro marker tests. The data provide insight into the relationship between genomic structure and attenuation of poliovirus type 1.

(A preliminary report of this work was presented at the meeting "Modern Approaches to Vaccines," Cold Spring Harbor, N.Y., September 1984 [27].)

MATERIALS AND METHODS

Construction of recombinant viruses. Both full-length cDNAs of the genomes of the virulent Mahoney and the attenuated Sabin 1 strains have been inserted into the *Eco*RI site of bacterial plasmid pBR325 by using *Eco*RI linkers (28, 36). The cloned cDNAs from both Mahoney and Sabin viruses are infectious in mammalian cells and are designated here as pVM(1)pDS306(25) and pVS(1)IC-0(25), respectively. The Mahoney and Sabin viruses [PV1(M)pDS306 and PV1(Sab)IC-0, respectively; Fig. 1] produced in HeLa S3 cells transfected with these two infectious cDNA clones showed biochemical and biological characteristics of the corresponding authentic parental virus strains previously (17, 28, 36) and in this study, as described below.

"Allele-replacement" (17) experiments were carried out on these two DNA clones by using restriction endonuclease sites of *Kpn*I, *Aat*II, *Pst*I, and *Bgl*II. As a result, seven different infectious cDNA clones from segments of the Mahoney and the Sabin 1 genomes were constructed and designated as pVSM(1)IC-1a(25), pVSM(1)IC-2a(25), pVSM(1)IC-2b(25), pVSM(1)IC-3a(25), pVSM(1)IC-3b(25), pVSM(1)IC-4a(25), and pVSM(1)IC-4b(25) (Fig. 1). HeLa S3 cells were transfected with 10 μg of closed circular forms of these recombinant cDNA clones per plastic dish (diameter, 6 cm), and the corresponding viruses, designated as PV1(SM)IC-1a, PV1(SM)IC-2a, PV1(SM)IC-2b, PV1(SM)IC-3a, PV1(SM)IC-3b, PV1(SM)IC-4a, and PV1(SM)IC-4b, respectively (Fig. 1), were recovered from the cells by the procedure previously reported (17, 28). All recombinant viruses were grown at 33.5 to 34°C, because every isolate showed temperature sensitivity as described below.

Virus multiplication. HeLa S3 monolayer cells were infected with the recombinant viruses at a multiplicity of infection of approximately 10^{-3}. After an incubation at 33.5 to 34°C for 4 days, the viruses were recovered by a freeze-thawing of the cells three times followed by a low-speed centrifugation to remove cell debris. The titer of the isolates contained in the supernatants was determined, and the virus was used directly for monkey neurovirulence tests and for other biological tests. These viruses were further passaged in HeLa S3 cell suspension cultures to produce high-titered virus stocks and used for the preparation of ^{32}P-labeled virus as described previously (17, 28). ^{32}P-labeled RNAs were prepared by phenol-chloroform extraction from purified ^{32}P-labeled virions and further purified by sucrose density gradient centrifugation as described previously (17, 28).

Monkey neurovirulence test. The titers of the viruses were determined by using primary-cultured cynomolgus monkey kidney cells (17, 28). Into both right and left thalamuses of each of the seronegative cynomolgus monkeys, 0.5 ml of virus suspension (10^7 50% tissue culture infective doses per ml) was inoculated (8). Monkeys showing severe clinical poliomyelitis were sacrificed at the peak of the disease. The other monkeys were sacrificed 17 days after the inoculation. A total of 38 sections (1 section each from cerebral cortex, thalamus, midbrain, cerebellum, and pons; 3 sections from medulla oblongata; 12 sections from cervical enlargement at different levels; 18 sections from lumbar enlargement at different levels) of the central nervous system were prepared to score the intensity of histological lesions. Lesion scores and spread values were estimated by established procedures (8).

Enzyme-linked immunosorbent assay. Antigenicities of viruses were tested by the modified method of enzyme-linked immunosorbent assay with neutralizing monoclonal antibodies specific to the Mahoney and Sabin 1 strains as described previously (17).

In vitro phenotypic marker tests. All in vitro phenotypic marker tests were performed with primary-cultured cynomolgus monkey kidney cells. The reproductive capacity at different temperatures (*rct* marker [21, 45]) of viruses was investigated by measuring the virus titer on the cells at a sodium bicarbonate concentration of 0.225% and at temperatures of 36, 39, 39.5, and 40°C after an incubation of 7 days as described previously (17, 28). For determination of plaque sizes (21, 45) produced by the different viruses, cells were infected and cultured under agar overlays at a sodium bicarbonate concentration of 0.225% at 36°C. The diameter of approximately 100 plaques, observed on days 4, 5, and 7 postinfection, were measured as described previously (17, 28). Delayed growth (*d* marker [21, 45]) of viruses in medium containing a low concentration of bicarbonate was investigated by measuring virus titers on the cells at 36°C and at

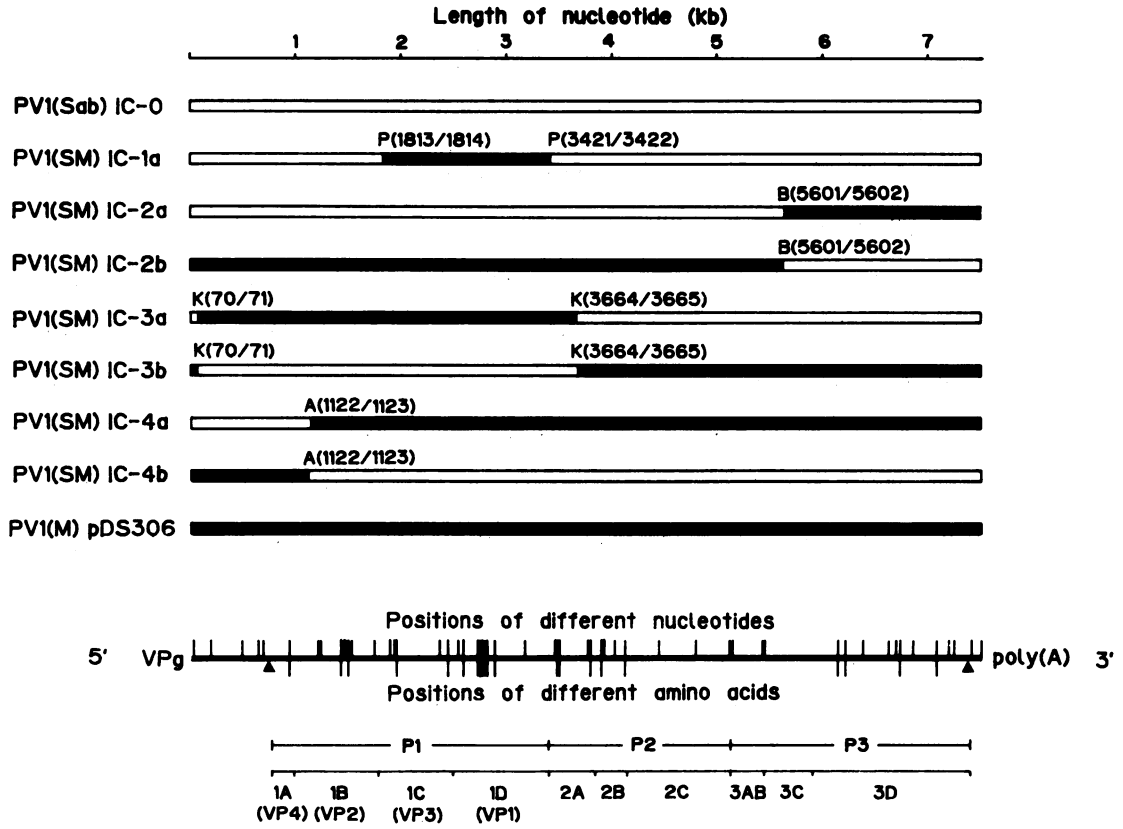

FIG. 1. Genome structure of recombinant type 1 polioviruses and locations of nucleotide and amino acid differences between the Mahoney and Sabin 1 strains. The expected genome structures of the recombinant viruses are shown by the combination of Sabin 1 (▭) and Mahoney (▬) sequences. K, A, P, and B represent cleavage sites of the restriction enzymes *Kpn*I, *Aat*II, *Pst*I, and *Bgl*II, respectively. Numbers in parentheses following the restriction sites indicate nucleotide positions from the 5′ end of the viral genome. Length of the entire genome of poliovirus type 1 is indicated at the top of the figure in kilobases (kb) from the 5′ terminus. Genomic RNA and its gene organization are shown at the bottom of the figure by using a recently adopted nomenclature (32). VPg is a small protein covalently attached to the 5′ end of the genome; poly(A) is 3′ terminal. The positions of initiation and termination (▲) of viral polyprotein synthesis are indicated. The locations of nucleotide and amino acid differences between the Mahoney and Sabin 1 strains are indicated by lines over and under the genome RNA, respectively. Recent sequence analysis of the infectious Sabin 1 cDNA clone revealed a cytosine residue at position 355, not a uracil as found originally (24). Thus, a total of 55 nucleotide differences exist between genomes of the Mahoney and Sabin 1 strains.

sodium bicarbonate concentrations of 0.255 and 0.03% after an incubation of 4 days as described previously (17, 28).

Analysis of DNA and RNA. Restriction fragments of DNA were analyzed and purified in 0.8 to 1.0% agarose gels as described previously (17, 28). Fingerprint analysis of RNase T_1 or RNase A digests of ^{32}P-labeled virion RNAs were carried out by two-dimensional polyacrylamide gel electrophoresis as described previously (17, 23, 28).

RESULTS

Construction of recombinant viruses from the Mahoney and the Sabin 1 parental strains. The total nucleotide sequences of the genomes of the virulent Mahoney and the corresponding attenuated Sabin strain of type 1 poliovirus have been determined (16, 24, 30). Comparison of the total nucleotide sequences has revealed 55 nucleotide substitutions (24, 40; Fig. 1) within the 7,441 total heteropolymeric bases. These nucleotide changes are scattered over the entire length of the genome and result in 21 amino acid replacements within the viral polyprotein (24, 40; see Fig. 1).

To analyze the effect of nucleotide and amino acid changes on the phenotype of the virus, we constructed seven different recombinant viruses from the Mahoney and Sabin 1 parental strains as described above. The expected genomic structures of the recombinant viruses are shown in Fig. 1.

Analysis of viral RNA. Our previous structural studies (22, 23) on the Mahoney and Sabin 1 genomes established the precise location of the large RNase T_1- and RNase A-resistant oligonucleotides on their respective viral genomes as well as their positions in two-dimensional RNA fingerprints. Accordingly, fingerprint analysis of the RNAs prepared from the viruses recovered from DNA-transfected cells was performed. Two-dimensional polyacrylamide gel electrophoresis patterns were then compared (Fig. 2). The patterns of the PV1(M)pDS306 and PV1(Sab)IC-0 genomes are identical to the established patterns (23) of the Mahoney and Sabin 1 genomes (Fig. 2A-D). Large oligonucleotides represented by spots 9, 30, 37, 1′, and 15′ are specific to the Mahoney genome, and oligonucleotides 9(LSc), a(LSc), 37(LSc), 1′(LSc), and 15′(LSc) are specific to the Sabin 1 genome (spots are numbered roughly by size; a number with a prime corresponds to an oligonucleotide produced by RNase A). The patterns for these strain-specific oligonucleotides differ from isolate to isolate, whereas the spots expected to be common to both strains show no variation in any of the fingerprints. Given the known positions on the viral genome of the large strain-specific oligonucleotides, the

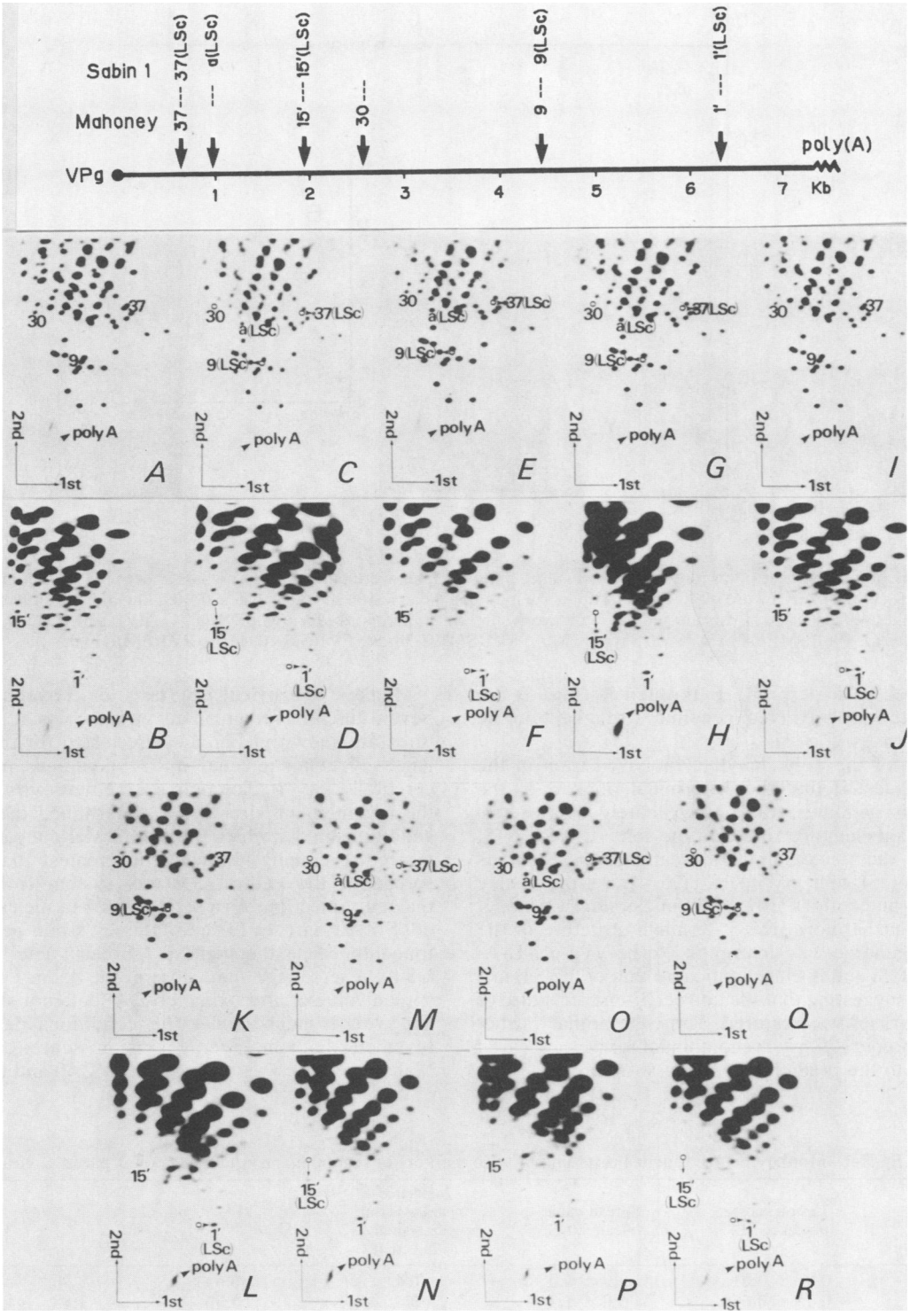

FIG. 2. Fingerprint analysis of genomes of the recombinant viruses and their parent viruses. Fingerprint analyses were carried out after digestion of the RNA with RNase T_1 (A, C, E, G, I, K, M, O, and Q) or RNase A (B, D, F, H, J, L, N, P, and R) and two-dimensional polyacrylamide gel electrophoresis as described in Materials and Methods. Fingerprints correspond to the following viruses: PV1(M)pDS306 (A and B), PV1(Sab)IC-0 (C and D), PV1(SM)IC-1a (E and F), PV1(SM)IC-2a (G and H), PV1(SM)IC-2b (I and J), PV1(SM)IC-3a (K and L), PV1(SM)IC-3b (M and N), PV1(SM)IC-4a (O and P), and PV1(SM)IC-4b (Q and R). Genomic RNA (marked in kilobases [kb] from the 5′ terminus) is shown at the top of the figure. Established map positions of unique spots corresponding to the Mahoney and Sabin 1 strains are indicated by lines above the genomic RNA. The nomenclature of the spots is as reported by Nomoto et al. (23). Mahoney-specific spots missing in the patterns of fingerprints (○) are indicated, as are shifts of spots (arrows or small arrowheads).

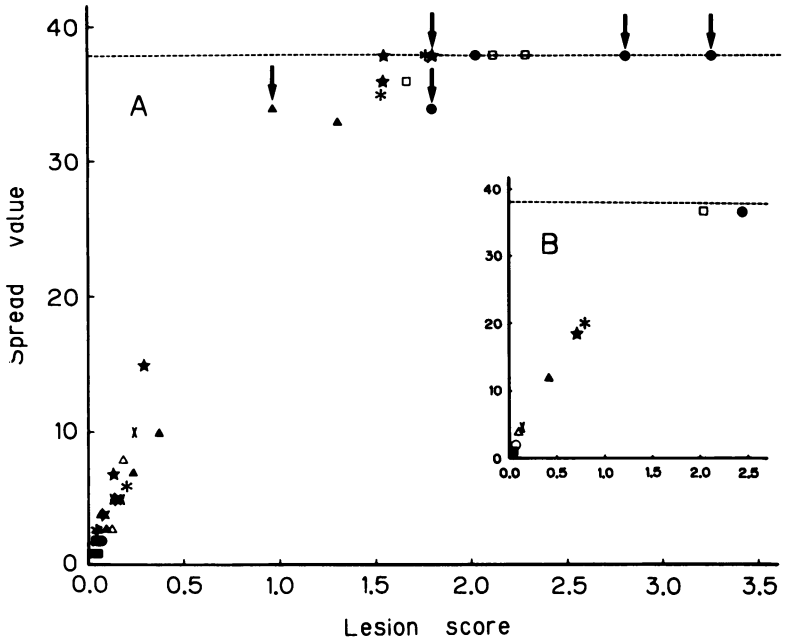

FIG. 3. Correlation between lesion score and spread value. Spread values were plotted versus lesion scores for each monkey (A), and the mean spread values of each virus were plotted versus the corresponding mean lesion scores (B). The dotted line indicates the maximum spread value, 38.0. Monkeys showing paralysis are indicated by arrows in (A). Symbols: ●, PV1(M)pDS306; ○, PV1(Sab)IC-0; X, PV1(SM)IC-1a; ■, PV1(SM)IC-2a; □, PV1(SM)IC-2b; ▲, PV1(SM)IC-3a; △, PV1(SM)IC-3b; ★, PV1(SM)IC-4a; *, PV1(SM)IC-4b.

results displayed in fingerprints E through R (Fig. 2) are compatible with the predicted genomic structures of the recombinant viruses (Fig. 1).

The position of the polyadenylate [poly(A)] tract in the fingerprints suggested that the 3'-terminal poly(A) of the recovered virus genomes was approximately 60 residues long (i.e., the same length as in wild-type poliovirus RNA [3, 25]), although the deoxyadenylate segments in plasmids pVM(1)pDS306(25) and pVS(1)IC-0(25) were previously shown to be 84 nucleotides (36) and 11 nucleotides (28) long, respectively. Furthermore, the 5'-terminal structure of the PV1(Sab)IC-0 genome was determined to be VPg-pU-U-A-A-A-A-C-A-G (28) and is thus identical to that of the Sabin 1 strain (24, 25), suggesting that the correct 5'-end structure of all recovered viruses was restored. Thus, the primary structures of the genomic RNAs of recombinant viruses appeared to be identical to the predicted genomic structures.

Monkey neurovirulence tests on recombinant viruses. Seven different recombinant viruses, whose genome structures are shown in Fig. 1, were tested for their neurovirulence by being injected into cynomolgus monkeys intracerebrally (8, 44). To compare the neurovirulence levels of the recombinant viruses, we determined the experimental conditions under which the parent Mahoney strain (used for molecular cloning) showed the greatest extent of lesion-spread in the central nervous system from the site of thalamus injection. As a result, a 50% tissue culture infective dose of 10^7 viruses of the Mahoney strain per monkey was found to cause the highest "spread value" in this study (38.0), whereas the same dose of the Sabin 1 strain caused a spread value of approximately 2.0 (data not shown). Preliminary experiments under the conditions described above revealed that the average lesion score caused by the Mahoney strain was approximately 2.50 and that that of the

TABLE 1. Monkey neurovirulence tests and in vitro markers of recombinant viruses and their parental viruses

Virus	Lesion score (mean ± SE)	Spread value (mean ± SE)	Incidence of paralysis (no. paralyzed/no. injected)	*rct*[a]	*d*[b]	Plaque size (mm in diameter)[c]
PV1(Sab)IC-0	0.07 ± 0.01	2.0 ± 0.0	0/4	>6.64	3.96	7.0
PV1(SM)IC-1a	0.15 ± 0.05	5.0 ± 1.9	0/4	5.70	1.88	8.5
PV1(SM)IC-2a	0.05 ± 0.01	1.5 ± 0.3	0/4	4.09	4.26	8.5
PV1(SM)IC-2b	2.03 ± 0.38	37.3 ± 0.6	0/4	2.77	0.66	14.5
PV1(SM)IC-3a	0.42 ± 0.17	11.1 ± 4.7	1/8	2.36	0.27	13.5
PV1(SM)IC-3b	0.11 ± 0.04	4.3 ± 1.3	0/4	4.73	4.63	6.5
PV1(SM)IC-4a	0.72 ± 0.27	18.4 ± 5.7	1/8	3.55	1.06	14.0
PV1(SM)IC-4b	0.80 ± 0.27	20.1 ± 6.1	0/8	5.09	3.18	4.5
PV1(M)pDS306	2.48 ± 0.34	37.0 ± 1.0	3/4	0.56	0.07	16.0

[a] *rct* marker values shown here are the logarithmic differences of virus titers obtained at 36 and 39.5°C (except for pV1(M)pDS306, for which the *rct* marker values of titers obtained at 36 and 40°C are given) (Fig. 4).
[b] *d* marker values shown here are the logarithmic differences of virus titers obtained at two different sodium bicarbonate concentrations (Fig. 6).
[c] Plaque size displayed on day 5 of growth (Fig. 5).

authentic Sabin 1 strain (the vaccine reference used in the Japan Poliomyelitis Research Institute) was always less than 0.10 (data not shown). The viruses recovered from cells transfected with pVM(1)pDS306(25) and pVS(1)IC-0(25) showed almost the same values as the corresponding parent strains (Table 1). Thus, a 50% tissue culture infective dose of 10^7 recombinant viruses was injected into a cynomolgus monkey intracerebrally for each neurovirulence test.

To determine the correlation of the parameters of neurovirulence, the spread value versus the lesion score was plotted for each monkey used in the neurovirulence tests; the monkeys that showed paralysis are indicated by arrows (Fig. 3A). A strong correlation between the spread values and lesion scores was observed in every monkey. The results indicated that the virus was able to spread more extensively through the central nervous system from the thalamus when it caused more lesions. The results also suggested that the maximum spread value was observed after infection with virus that caused a lesion score of approximately 1.70. Paralysis was observed only in monkeys which showed high spread values. However, the incidence of paralysis (number of monkeys paralyzed per number of monkeys injected) was not always a conclusive parameter of neurovirulence in our experiments. Although the inoculation with some of the virus isolates caused considerable fluctuation in lesion scores and spread values, any occurrence of high average values in both parameters was strong indication that the virus was not attenuated. The average lesion scores and spread values were therefore employed as the main parameters for estimating the neurovirulence of viruses. For recombinant viruses PV1(SM)IC-3a, PV1(SM)IC-4a, and PV1(SM)IC-4b, eight monkeys were used for the tests (Table 1), since lesion scores and spread values of these viruses varied considerably (Fig. 3A). Four monkeys were used for neurovirulence tests of other recombinant viruses.

The resulting mean values of lesion scores and spread values for each recombinant virus are shown in Table 1, and the numbers are plotted in Fig. 3B. Virus PV1(SM)IC-2b showed the highest lesion score and spread value among the recombinants, although both lesion score and incidence of paralysis were clearly lower than those of the parental neurovirulent strain PV1(M)pDS306. In contrast, recombinant PV1(SM)IC-2a showed almost the same phenotype as that of the attenuated PV1(Sab)IC-0 parental virus. These results suggest that the major determinants of neurovirulence reside in the Mahoney genome represented by the *Bgl*II fragment of nucleotide positions 1 to 5601. This conclusion is compatible with the observations of Agol et al. (2). Although virus PV1(SM)IC-3a was found to cause paralysis in one out of eight monkeys, the average lesion score for this recombinant as measured in eight monkeys was 0.42 ± 0.17 and thus was very low. It is therefore surprising that the genetic information comprising the entire capsid protein region is not sufficient to render the virus fully neurovirulent. Considering that PV1(SM)IC-3b also showed low neurovirulence (with a lesion score of 0.11 ± 0.04) (Table 1), it appears that all Mahoney genome segments up to nucleotide position 5601 are required for the expression of strong neurovirulence of type 1 poliovirus. In addition, the results with recombinant viruses PV1(SM)IC-4a and PV1(SM)IC-4b showed a relatively low level of neurovirulence (Table 1). This result indicates that both Mahoney genome segments, defined by *Aat*II fragments (nucleotide positions 1 to 1122 and 1123 to 7441), contain information involved in the expression of the neurovirulence phenotype. These data lead us to conclude that multiple determinants are involved in the expression of neurovirulence of type 1 poliovirus and that these determinants are encoded at multiple loci along a large portion of the viral genome.

It should be noted that the nucleotide sequence upstream of the *Aat*II restriction site (nucleotide positions 1 to 1122) represents mainly the noncoding region of poliovirus RNA, since the translation of the polyprotein commences at nucleotide position 743 (7). Nucleotides 743 to 1122 code for capsid protein VP4 and a part of capsid protein VP2 (Fig. 1). Region 743 to 1122 harbors only one nucleotide difference between Mahoney and Sabin 1, resulting in one amino acid change in VP4 (Ala→Ser [24]). The amino acid at this position (residue 65) in VP4, an internal polypeptide of the virion (4, 18, 43) of both the Sabin 2 and Sabin 3 strains, is Ala and is thus the same as that of the virulent Mahoney strain (26, 40). On the basis of these considerations, the one amino acid difference in VP4 between Mahoney and Sabin 1 may contribute little to phenotypes of virulence or attenuation, although this will have to be tested with more recombinants. Thus, it appears that the mutations in the 5′ noncoding region of the Sabin 1 strain significantly influence the attenuation phenotype. The mechanism for this phenomenon is unknown but could be a modulation in one or all of the following steps in viral proliferation: initiation of protein synthesis, RNA replication, or morphogenesis. Such changes in viral proliferation become plausible, considering that the 5′-terminal 1,000 bases of Mahoney and Sabin 1 RNAs assume strikingly different structures when folded by the computational methods of Jacobson et al. (14) (unpublished data). Indeed, the genomes of type 1 Mahoney and type 1 Sabin differ in their efficiencies in in vitro translation (38a). Moreover, a single base change in the 5′ untranslated region of poliovirus type 3 has been correlated with the attenuation phenotype of this strain (12).

Antigenicity. The genomic locus represented by the *Pst*I fragment (nucleotide positions 1814 to 3421) codes for capsid protein VP1 and most of VP3 (Fig. 1). These polypeptides are predominantly external proteins of the virion particle (9, 41, 43) that carry a number of antigenic and immunological determinants (10) and also harbor the highest degree of amino acid substitutions between the Mahoney and Sabin 1 strains (24) (Fig. 1). In fact, PV1(SM)IC-1a, which contains only the Mahoney sequence coding for VP1 and most of VP3, showed antigenicity specific to the Mahoney strain as reported previously (17). In addition, all recombinant viruses that have the Mahoney sequence in the same genomic locus as that of PV1(SM)IC-1a (i.e., PV1(SM)IC-2b, PV1(SM)IC-3a, and PV1(SM)IC-4a) showed Mahoney-specific antigenicity. Recombinant viruses PV1(SM)IC-2a, PV1(SM)IC-3b, and PV1(SM)IC-4b showed Sabin 1-specific antigenicity in an enzyme-linked immunosorbent assay (17) with strain-specific neutralizing monoclonal antibodies (data not shown). These results clearly demonstrate that the genomic sequence corresponding to the *Pst*I fragment (nucleotide positions 1814 to 3421) is responsible for the strain-specific antigenicity.

Temperature sensitivity. The Sabin 1 strain is considered to be a multiple-step temperature-sensitive mutant (13). The temperature-sensitive phenotype of the Sabin strain is used as an in vitro marker to estimate the quality of live vaccines. The *rct* marker test for this phenotype is one of the most reliable in vitro marker tests of poliovirus vaccines (21). The mutations that influence the temperature sensitivity of the Sabin 1 strain have not yet been defined. To identify the genomic loci contributing to the temperature sensitivity, recombinant viruses were assayed for the temperature-

sensitive phenotype by measurement of virus titers at 36, 39, 39.5, and 40°C. The results reveal that virus isolates PV1(Sab)IC-0 and PV1(M)pDS306 showed phenotypes similar to those of the authentic Sabin 1 and Mahoney strains (17, 28), respectively, whereas every recombinant virus showed a phenotype intermediate to those of the two parent strains (Fig. 4). These results strongly suggest that there are multiple determinants of temperature sensitivity for the Sabin 1 virus.

Recombinant virus PV1(SM)IC-1a is slightly less temperature sensitive than is virus PV1(Sab)IC-0, whereas the recombinant virus PV1(SM)IC-3a has acquired a temperature-resistant phenotype like that of the parent Mahoney virus (Fig. 4). PV1(SM)IC-1a has capsid proteins VP1 and most of VP3 derived from the Mahoney strain, and PV1(SM)IC-3a has coat protein derived entirely from the Mahoney strain. These results are compatible with the observation that the assembly of Sabin virus capsid proteins into capsomeres includes a temperature-sensitive step (13). Recombinant virus PV1(SM)IC-2a also has lost some of the temperature sensitivity of the PV1(Sab)IC-0 virus, whereas its reciprocal recombinant, PV1(SM)IC-2b, is partially temperature sensitive compared with the PV1(M)pDS306 parent virus. These results suggest that a mutation(s) in the 3'-terminal 1,840 nucleotides of the Sabin 1 genome (i.e., the sequences downstream from the *Bgl*II site at nucleotide 5601) partially influences the temperature sensitivity. Mutations in that genomic locus result in four amino acid changes in the viral RNA polymerase 3D (24) (Fig. 1). In addition, two nucleotide substitutions were observed in the 3' noncoding sequence of the Sabin 1 genome (24). One or more of these mutations must influence the temperature-sensitive phenotype. The mutation(s) in this 3'-terminal region of the Sabin 1 genome also appears to slightly affect neurovirulence (compare the lesion scores and incidence of paralysis of PV1(SM)IC-2b and PV1(M)pDS306 in Table 1). Similarly, comparison of the results shown in Fig. 4 of recombinant viruses PV1(SM)IC-4a and PV1(SM)IC-4b with those of the parental PV1(M)pDS306 and PV1(Sab)IC-0 viruses, respectively, revealed that the 5'-proximal segment of 1,122 nucleotides, most of which comprise the noncoding sequence, also included mutations that influence viral temperature sensitivity. Mutations in this same region of the genome significantly affected viral neurovirulence (Table 1). Thus, determinants of temperature sensitivity, like those of neurovirulence, are located across the entire poliovirus genome.

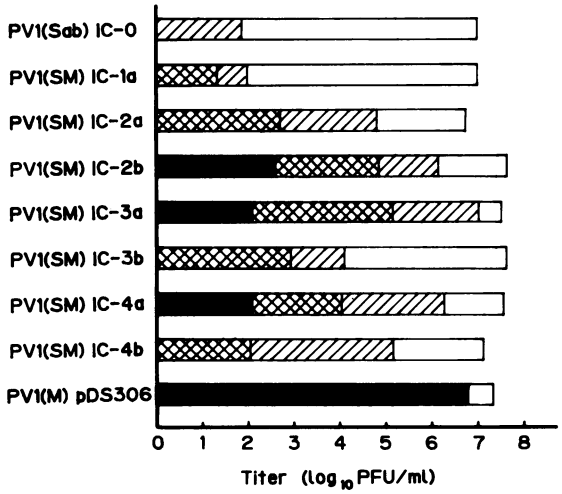

FIG. 4. Reproductive capacity of recombinant viruses and their parent viruses at different temperatures. Titers of viruses were measured after viruses were grown for 7 days in primary-cultured cynomolgus monkey kidney cells at a sodium bicarbonate concentration of 0.225% and at temperatures of 36 (□), 39 (▨), 39.5 (▩), and 40°C (■), as described previously (17, 28). Titers of viruses at different temperatures are indicated by the lengths of the bands.

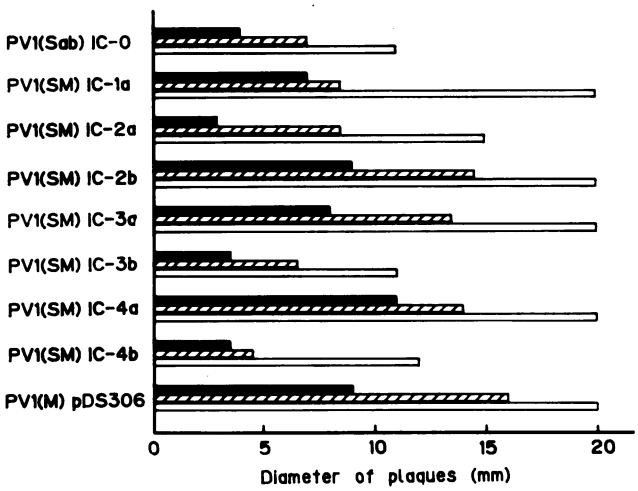

FIG. 5. Plaque sizes of recombinant viruses and their parent viruses. Viruses prepared as described in Materials and Methods were used to infect primary-cultured cynomolgus monkey kidney cells. The cultures were then incubated under agar overlays at a sodium bicarbonate concentration of 0.225% at 36°C as described previously (17, 28). The diameters of approximately 100 plaques, displayed on days 4 (■), 5 (▨), and 7 (□), were measured for each virus, and the average sizes are shown in millimeters. The range of nearly all plaques was ±2 mm of the average size.

Plaque size. Plaque size is also one of the marker tests used as an indication of the rate of viral multiplication, and it might correlate with viral virulence (33). Indeed, the plaque size of the Mahoney strain is larger than that of the Sabin 1 strain. Therefore, we investigated the extent to which the allele replacements in our recombinant viruses, measured at 36°C, influence plaque size. The size of plaques increased during the indicated incubation period to an average maximum diameter of 20 mm (for viruses with Mahoney growth properties) (Fig. 5). Comparison of the average size of approximately 100 plaques for each parental and recombinant virus revealed that infection with PV1(Sab)IC-0, PV1(SM)IC-2a, PV1(SM)IC-3b, PV1(SM)IC-4b, and, to a lesser extent, PV1(SM)IC-1a yielded relatively small plaques compared with those produced by the other viruses (Fig. 5). The molecular basis for this phenomenon is unknown. However, the small-plaque phenotype might be a reflection of virus aggregation that may occur under the conditions used for our plaque assays. Considering that the Sabin 1 virus forms aggregates more readily than the Mahoney strain under low ionic strength or low pH or both (39) makes this possibility a plausible one, because the small-plaque recombinant viruses have derived most of their capsid proteins from the Sabin 1 strain. It should be noted that the small-plaque phenotype of recombinant virus PV1(SM)IC-1a, which derives most of its surface antigens from the Mahoney virus, is unstable upon repeated passages and will produce large-plaque variants after several passages in HeLa S3 cells

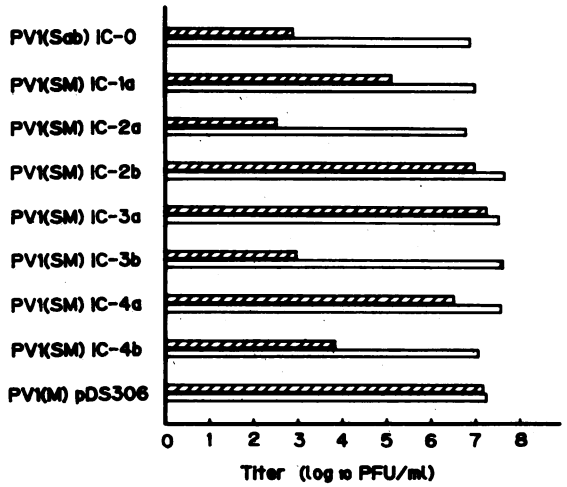

FIG. 6. Delayed growth of recombinant viruses and their parent viruses in medium containing low bicarbonate concentrations. Viruses prepared as described in Materials and Methods were used to infect primary-cultured cynomolgus monkey kidney cells in media at a sodium bicarbonate concentration of 0.225%. The cell cultures were incubated at 36°C and at sodium bicarbonate concentrations of 0.225 and 0.03% for 4 days, after which plaques were counted (17, 28). Virus titers at sodium bicarbonate concentrations of 0.225 (▭) and 0.03% (▨) are indicated by the lengths of the bands.

as described previously (17). It is possible that the small-plaque phenotype of PV1(SM)IC-1a correlates with determinants other than aggregation. For example, the efficiency of a certain step of the viral replication cycle (such as capsid assembly or protein processing) may be reduced (17), resulting in a slightly lower rate of viral multiplication. In any event, the small-plaque phenotype appears to be due to the expression of the Sabin 1 sequence corresponding to the *Aat*II-*Kpn*I fragment (nucleotide position 1123 to 3664).

Bicarbonate concentration dependency. The plaque-forming ability of the attenuated Sabin 1 strain is sensitive to reduced concentrations of bicarbonate in the agar overlay, but that of the virulent Mahoney strain is not (21). This characteristic of the Sabin strain is often used in the in vitro *d* marker test of the virus that may correlate with the attenuated phenotype. As with other phenotypes, the molecular basis of the *d* phenotype is unknown (42). To determine which regions of the genome influence the *d* phenotype of the Sabin 1 strain, *d* marker tests were performed on the parental and recombinant polioviruses (Fig. 6). Virus titers were measured under agar overlays containing sodium bicarbonate concentrations of 0.03% or 0.225%. The titers of virus strains PV1(Sab)IC-0, PV1(SM)IC-2a, PV1(SM)IC-3b, and PV1(SM)IC-4b were dramatically reduced when the concentration of sodium bicarbonate was reduced from 0.225 to 0.03% (Fig. 6). The common genome segment derived from the Sabin 1 virus among those virus strains sensitive to the reduced bicarbonate concentration is the *Aat*II-*Kpn*I fragment (nucleotides 1123 to 3664) that encodes the capsid proteins (Fig. 1). This region of the genome is the same locus suggested for that of the small-plaque phenotype, as described above. We therefore suggest that there are common determinants influencing both in vitro phenotypes of plaque size and *d* marker and that these determinants reside in the capsid proteins of the Sabin 1 strain.

Correlation between neurovirulence and in vitro pheno-

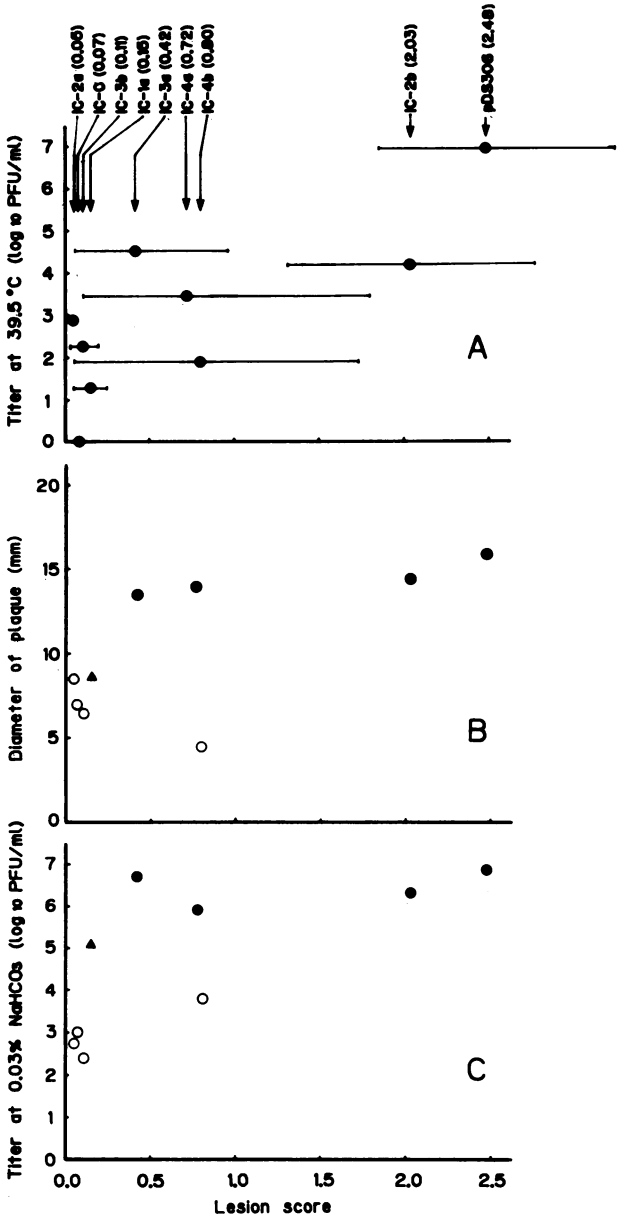

FIG. 7. Correlation between lesion scores and in vitro phenotypes. Virus titers at 39.5°C (A), diameters of plaques on day 5 (B), and virus titers at a sodium bicarbonate concentration of 0.03% (C) are plotted versus mean lesion scores for each virus. The range of lesion scores is indicated by lines in (A). The nomenclature of virus strains, indicated at the top of the figure, was modified slightly for technical reasons; PV1(M), PV1(Sab), and PV1(SM) were omitted. The numbers in parentheses represent mean lesion scores of the corresponding viruses as shown in Table 1. (A) Data taken from Fig. 4. Virus titers at 39.5°C used here, however, are calculated assuming that the titer of every virus at 36°C is 10^7 PFU/ml. (B) Plaque sizes observed on day 5 were taken from data shown in Fig. 5. Viruses encoding mostly Mahoney (●) and Sabin (○) capsid proteins are indicated. ▲, PV1(SM)IC-1a. (C) Data taken from Fig. 6. Virus titers at a sodium bicarbonate concentration of 0.02% were calculated assuming that the titer of every virus at 0.225% was 10^7 PFU/ml.

types. As was discussed before, the average lesion score resulting from a cerebral poliovirus inoculation is a parameter of the neurovirulence of the strain. We have, therefore, attempted to correlate lesion scores of the parental and recombinant viruses with the following biological characteristics: (i) antigenicity, (ii) temperature sensitivity (*rct* marker), (iii) plaque size, and (iv) bicarbonate concentration dependency (*d* marker).

Recombinant virus PV1(SM)IC-1a had the antigenicity of the virulent Mahoney strain (17), yet the lesion score caused by the recombinant virus was considerably lower than that of the parent Mahoney virus (Table 1). Moreover, the PV1(SM)IC-4a and PV1(SM)IC-4b recombinants had the Mahoney- and Sabin 1-specific antigenicities, respectively, and both of these virus strains showed almost the same lesion scores. These results suggest that surface parameters such as antigenicity and immunogenicity are not correlated with the lesion scores. This is supported by studies with poliovirus type 1 mutants selected either by monoclonal antibodies (6) or at supraoptimal temperatures (R. Crainic, B. Blondel, A. Candrea, G. Duffrais, and F. Horand, Dev. Biol. Stand., in press).

A correlation of the other three in vitro phenotypes with the lesion scores is shown in Fig. 7, in which virus titers at 39.5°C, plaque size as measured on day 5 after infection, and virus titers at a bicarbonate concentration of 0.03% were plotted versus the corresponding lesion scores for each virus. Some parameters of the in vitro markers have also been listed (Table 1) to compare them with the parameters of lesion score, spread value, and incidence of paralysis. Some correlation between *rct* at 39.5°C and lesion score was observed (Fig. 7A). These data suggest that the temperature sensitivity of the virus strains is one of the most important factors contributing to the extent of lesion scores. The data shown in Fig. 7A, however, do not exclude the possibility that other biological characteristics of these strains may also affect the lesion score.

In contrast to the *rct* marker, the average plaque size (measured on day 5 postinfection) did not show a significant correlation when plotted versus the lesion score (Fig. 7B). As pointed out above, the viruses with coding sequences for the Sabin-specific capsid proteins showed the small-plaque phenotype, whereas those with coding sequences for the Mahoney-specific capsid proteins showed the large-plaque phenotype. Strains displaying similar plaque sizes, however, showed wide ranges of lesion scores, a phenomenon particularly evident for viruses with Mahoney capsid proteins (Fig. 7B). The capsid protein region, therefore, appears to influence plaque size but not lesion score. Although the strains with the Sabin capsid proteins generally showed low lesion scores, we conclude that plaque size is clearly not a reliable marker to estimate neurovirulence or attenuation.

A very similar pattern emerged when virus titers obtained at low concentration of bicarbonate were plotted against lesion scores (Fig. 7C). This observation supports the notion that the *d* marker phenotype is determined largely by the capsid proteins of the corresponding virus strain. Strain PV1(Sab)IC-0 and recombinants PV1(SM)IC-2a, PV1(SM)IC-3b, and PV1(SM)IC-4b, all of these viruses having Sabin-specific capsid proteins, grew by a 3- to 4-log difference to yields at the two different bicarbonate concentrations (Fig. 6). To our surprise, however, no differences in the burst sizes of the viruses at the two concentrations of bicarbonate were observed (data not shown). This observation suggests that the burst size does not influence the virus titers found under the two different conditions. Whether the *d* marker phenotype is determined by properties of the virus or of the indicator cells or of both remains to be seen.

DISCUSSION

Knowledge of the total nucleotide sequences of the genomes of the virulent Mahoney (16, 30) and the attenuated Sabin 1 (24) strains of poliovirus and the availability of infectious cDNA clones of both strains (28, 31, 36) suggested the use of a molecular genetic approach to identify mutations which influence the biological differences between the two strains (17). In the studies reported here, biological tests were carried out on recombinant viruses constructed in vitro by allele replacement of the genome segments between both strains. The results demonstrate that specific genomic loci contribute to the biological differences between the two strains of virus, including viral neurovirulence. Thus, the work presented here has provided an important example of how recombinant DNA technology can be used to investigate the relationship of gene structure and functions in poliovirus. Moreover, 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.

The results of monkey neurovirulence tests on the recombinant viruses revealed that many of the numerous mutations discovered in the genome of the Sabin 1 strain influence the phenotype of viral attenuation. The loci influencing attenuation are spread over wide areas of the viral genome, including the 5′ noncoding region. These conditions are compatible with the observation that the Sabin 1 virus is a relatively stable vaccine, as multiple mutations would be required to regain the neurovirulent phenotype. Most vaccine-associated cases of poliomyelitis appear to involve vaccine strains of type 2 and type 3 polioviruses (Sabin 2 and Sabin 3 strains, respectively) (5, 15, 20, 46). It came as no surprise, therefore, that sequence studies of the genomes of the neurovirulent poliovirus, type 3 (Leon), and its attenuated derivative, the Sabin 3 strain, revealed only 10 nucleotide differences; of these mutations, only three led to amino acid replacements (37, 38, 40). Here, we have shown that surface parameters such as antigenicity and immunogenicity are not the main determinants of the attenuated phenotype of the Sabin 1 strain (Table 1 and Fig. 1). More stable (and hence safer) vaccine strains of type 2 and type 3 polioviruses might therefore be constructed in vitro by the replacement of only the sequence encoding the antigenic determinants of the Sabin 1 genome by the corresponding sequences of the type 2 and type 3 genomes, respectively. Although this strategy of vaccine development appears to be straightforward, it is not known whether allele replacements between the different types of polioviruses yield viable viruses. As reported previously, recombinant virus strain PV1(SM)IC-1a, whose genome contains only Sabin 1 sequences except for the genome locus encoding the antigenic determinants, was observed to be an unstable virus (17).

Lesion scores correlated to some extent with the *rct* marker, a test being a measure of viral multiplication at temperatures above 36°C. The level of viral multiplication in the central nervous system of monkeys may thus be an important factor for the expression of viral neurovirulence. Plaque size and *d* marker tests, however, did not provide reliable estimates of the attenuated phenotype or of the potential for neurovirulence by the recombinant viruses. All in all, none of the in vitro markers analyzed in this study

were found to be a perfect reflection of the biological character of the virus. This observation correlates well with the recent analysis of an antibody-selected mutant of the Mahoney strain of poliovirus that had a single amino acid substitution in VP3 (Thr→Lys), a point mutation causing an antigenic shift from the Mahoney to the Sabin 1 phenotype (6). Although the mutant was neutralized by all Sabin 1-specific monoclonal antibodies, its neurovirulence phenotype remained entirely that of the parental Mahoney strain (6). Our results suggest that expression of the attenuated phenotype of the Sabin 1 strain requires a number of biological characteristics. Establishment of additional in vitro marker tests and the experimental conditions to make in vitro tests more reliable is desired and is currently in progress.

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