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Genetic Basis of Attenuation of the Sabin Type 3 Oral Poliovirus Vaccine

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The poliovirus type 3 Sabin oral poliovirus vaccine strain P3/Leon/12a$_1$b differs in nucleotide sequence from its neurovirulent progenitor P3/Leon/37 by just 10 point mutations. The contribution of each mutation to the attenuation phenotype of the vaccine strain was determined by the construction of a series of recombinant viruses from infectious cDNA clones. The neurovirulence testing of recombinant viruses indicated that the attenuation phenotype is determined by just two point mutations: a C to U in the noncoding region at position 472 and a C to U at nucleotide 2034 which results in a serine-to-phenylalanine amino acid substitution in the structural protein VP3.

Poliomyelitis is an acute paralytic disease of humans caused by three distinct serotypes of poliovirus. Over the past 25 to 30 years, the disease has been controlled in many developed countries by the use of the Sabin live-attenuated vaccine (3). This vaccine is based on attenuated strains of poliovirus which were derived empirically by passage of wild-type viruses in monkey tissue in vivo and in vitro (22, 23). The attenuated strains infect and induce immunity in humans without causing disease. Although the Sabin vaccine against poliomyelitis is arguably the safest and most effective live-virus vaccine currently in use, it has been shown that in a small number of cases, estimated as 0.84 cases per 1 million recipients, poliomyelitis may develop as a consequence of vaccination (3, 6, 13, 14, 18). Such disease is most frequently associated with the type 2 and type 3 vaccine strains and rarely with type 1 (3). The very good safety record of the vaccines is maintained by a rigorous program of testing in primates, which is expensive, laborious, and requires a high degree of technical expertise (29). An understanding of the molecular basis of attenuation of the Sabin vaccines could lead to improved production and control methods and to the development of modified vaccines incapable of reverting to neurovirulence.

Our previous work has concentrated on poliovirus type 3, the serotype most commonly implicated in vaccine-associated disease and that which has presented the most problems in vaccine control (3, 4). The genome of this virus is a single strand of positive-sense RNA of 7,432 bases (10, 20, 24). A 5′ noncoding region of 742 bases with a terminally linked protein (VPg) precedes a single open reading frame coding for the structural proteins which make up the virus particle (VP1 to VP4) and the nonstructural proteins involved in replication, including an RNA polymerase and two proteases (10). The RNA terminates in a 3′ noncoding region of 72 bases followed by a poly(A) tract of 40 to 100 bases. Comparison of the complete nucleotide sequences of the genomes of the Sabin type 3 vaccine strain P3/Leon/12a$_1$b and its neurovirulent progenitor P3/Leon/37 indicates that they differ by just 10 point mutations (Fig. 1) (26). Information on which of these might be responsible for the attenuation phenotype was obtained from the genome sequence of strain P3/119, a neurovirulent revertant of the vaccine isolated from a patient with a fatal case of vaccine-associated poliomyelitis (5). The data suggested that the mutations most likely to be important include a change in the 5′ noncoding region at position 472, one or more of the three mutations which affect the amino acid sequence of the polyprotein, and a mutation just prior to the poly(A) tract at position 7432 (5). Further evidence that the mutation at position 472 is important has come from a limited sequence analysis of other revertant strains and of excreted vaccine virus (2, 7). Definitive information on the contribution of each mutation to the attenuation phenotype, however, required the construction of strains in which their individual and combined effects could be assessed, an approach which has produced useful information on the poliovirus type 1 vaccine (11, 17, 19). Here we describe the construction and neurovirulence testing of recombinant viruses derived from infectious cDNAs (21) of the Sabin vaccine strain P3/Leon/12a$_1$b and its neurovirulent progenitor strain P3/Leon/37.

MATERIALS AND METHODS

Construction of recombinant viruses. The construction of full-length infectious cDNA clones derived from the vaccine progenitor P3/Leon/37 and the vaccine revertant P3/119 has been described previously (27). Similar methods were used to construct a full-length cDNA copy of the Sabin vaccine strain P3/Leon/12a$_1$b from partial cDNA clones in vector pAT153, generated as described previously (24). The first viable clone of this virus, designated pSabin/GDW, was later found by direct RNA sequence analysis of recovered virus to have a single base deletion at nucleotide 417 compared with RNA from stock P3/Leon/12a$_1$b virus and other cDNA clones. The pSabin/GDW clone was subsequently repaired by exchanging the 5′ region of the cDNA with the corresponding region from an independent partial clone. The repaired full-length cDNA was designated pS3/FLC. Both pSabin/GDW and pS3/FLC were used for the construction of recombinants. Recombinants were constructed by using convenient restriction endonuclease cleavage sites falling

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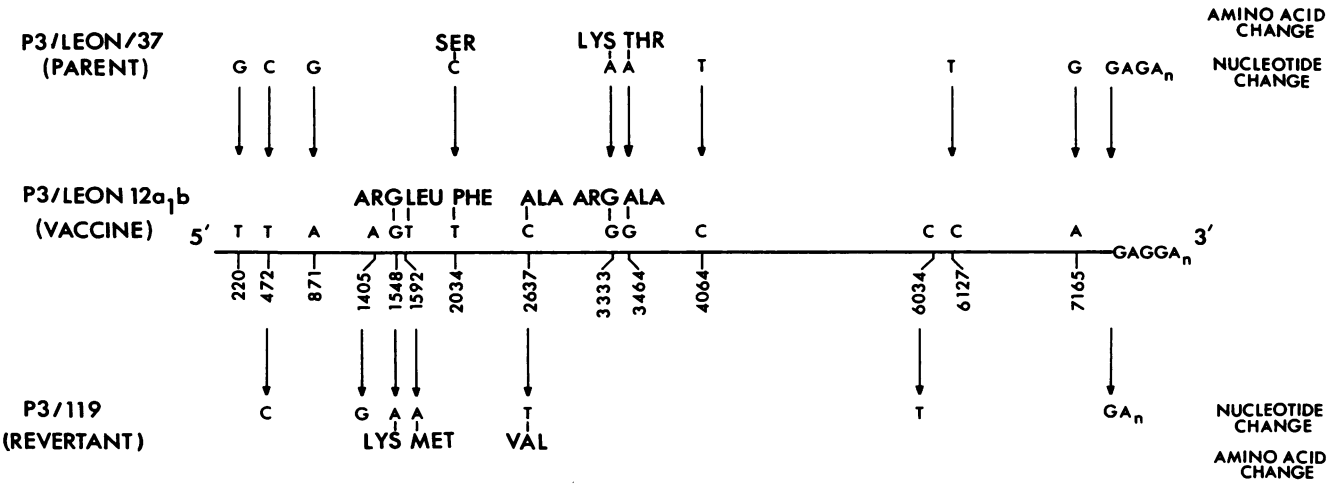

FIG. 1. Nucleotide sequence differences between the progenitor strain P3/Leon/37 (26), the Sabin type vaccine P3/Leon/12a$_1$b (24), and a vaccine revertant strain, P3/119 (5). There is some variability between cDNA clones of the virus at the 3' terminus just prior to the poly(A) tract (see text).

between the mutations considered to be important to the attenuation phenotype (5). Overlapping clones were digested with the required restriction enzymes under conditions specified by the manufacturer. After electrophoresis in agarose gels, the DNA was electroluted and purified by phenol extraction. The desired fragments were ligated with T4 DNA ligase in a 30-μl reaction mixture containing DNA (5 to 20 ng), 50 mM Tris hydrochloride (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and ligase (0.1 to 1 U). Ligated DNA was used to transform competent *Escherichia coli* JA221, and the resultant clones were characterized by restriction mapping. Recombinant plasmids were grown in 250 ml of medium and purified by CsCl gradient centrifugation (27).

Recovery and purification of recombinant viruses. Parental and recombinant viruses were recovered from full-length cDNAs by transfection of HEp-2C cells in culture (21). HEp-2C cells were grown to 60% confluence in 9-cm plastic dishes and transfected with approximately 20 μg of plasmid DNA by the calcium phosphate technique with a 20% glycerol shock. Cytopathic effect was observed after 5 to 7 days of incubation at 34°C, and virus stocks were prepared for sequence analysis and neurovirulence testing after one passage. Recombinant viruses were verified by direct RNA sequence analysis (8) by the primer extension method at positions in their genomes known to differ between the two parental strains (26).

Neurovirulence testing. Recovered parental and recombinant virus stocks were tested for neurovirulence by the standard World Health Organization-approved vaccine safety test, details of which have been described previously (25, 29).

RESULTS

Recovery of parental viruses from cloned cDNA. The cloning and assembly of a full-length DNA copy of the genome of the neurovirulent vaccine-progenitor strain, P3/Leon/37, has been described previously (27). The plasmid carrying this cDNA was designated pOLIO/Leon. Similar methods were used to construct full-length cDNAs of the vaccine virus P3/Leon/12a$_1$b as described in Materials and Methods. These plasmids were designated pSabin/GDW and pS3/FLC.

Viruses were recovered from plasmids pOLIO/Leon, pSabin/GDW, and pS3/FLC by transfection of HEp-2C cells in culture at 35°C by established techniques (21). The recovered viruses were then examined for neurovirulence by the standard World Health Organization-approved test for vaccine safety (25, 29) except that fewer animals were used per test.

Sabin/GDW and S3/FLC produced no clinical signs of poliomyelitis and gave low histological lesion scores and were thus very similar to standard preparations of the Sabin type 3 vaccine, P3/Leon/12a$_1$b (Fig. 2). Like the vaccine virus, they also failed to form plaques at 40°C (data not shown). In contrast, the recovered P3/Leon/37 produced severe clinical disease in all animals and gave high lesion scores (2.71) (Fig. 2), a result similar to that produced by our virus stocks of this wild strain (25). Recovered P3/Leon/37, like the original, was also able to form plaques at 40°C almost as efficiently as at 35°C (data not shown). These experiments confirmed that the viruses recovered from cDNA clones had phenotypes indistinguishable from those of the standard preparations of virus from which they were derived.

Construction of recombinant viruses between the wild-type progenitor strain P3/Leon/37 and the type 3 Sabin vaccine strain P3/Leon/12a$_1$b. Using convenient restriction endonuclease cleavage sites, we constructed recombinant cDNA clones in which segments of the genomes of the vaccine and progenitor genomes were exchanged. Initially, the recombinants were designed to contain the mutations considered to be important for attenuation (5), either singly or in combination, in the genome of the virulent virus P3/Leon/37. In later experiments, our strategy included the construction of cDNA clones in which attenuating mutations were removed from the genome of the vaccine virus.

The structure of the recombinant viruses is summarized in Fig. 2. Plasmid pSLR1 was constructed by using the unique *Sma*I site at nucleotide 2769, the sequence from the 5' terminus to position 2769 being derived from pSabin/GDW with the remainder from pOLIO/Leon (2). pST/L was constructed by exchanging the region of the pSLR1 cDNA from the *Aat*II site at position 1809 to the *Sma*I site at position 2769 for the equivalent region of pOLIO/Leon. Ten further recombinant cDNA clones were constructed in a similar manner with appropriate restriction endonucleases. Of those containing the 5'-terminal region of the vaccine strain, pST/L and pSLR1 were constructed from pSabin/GDW and

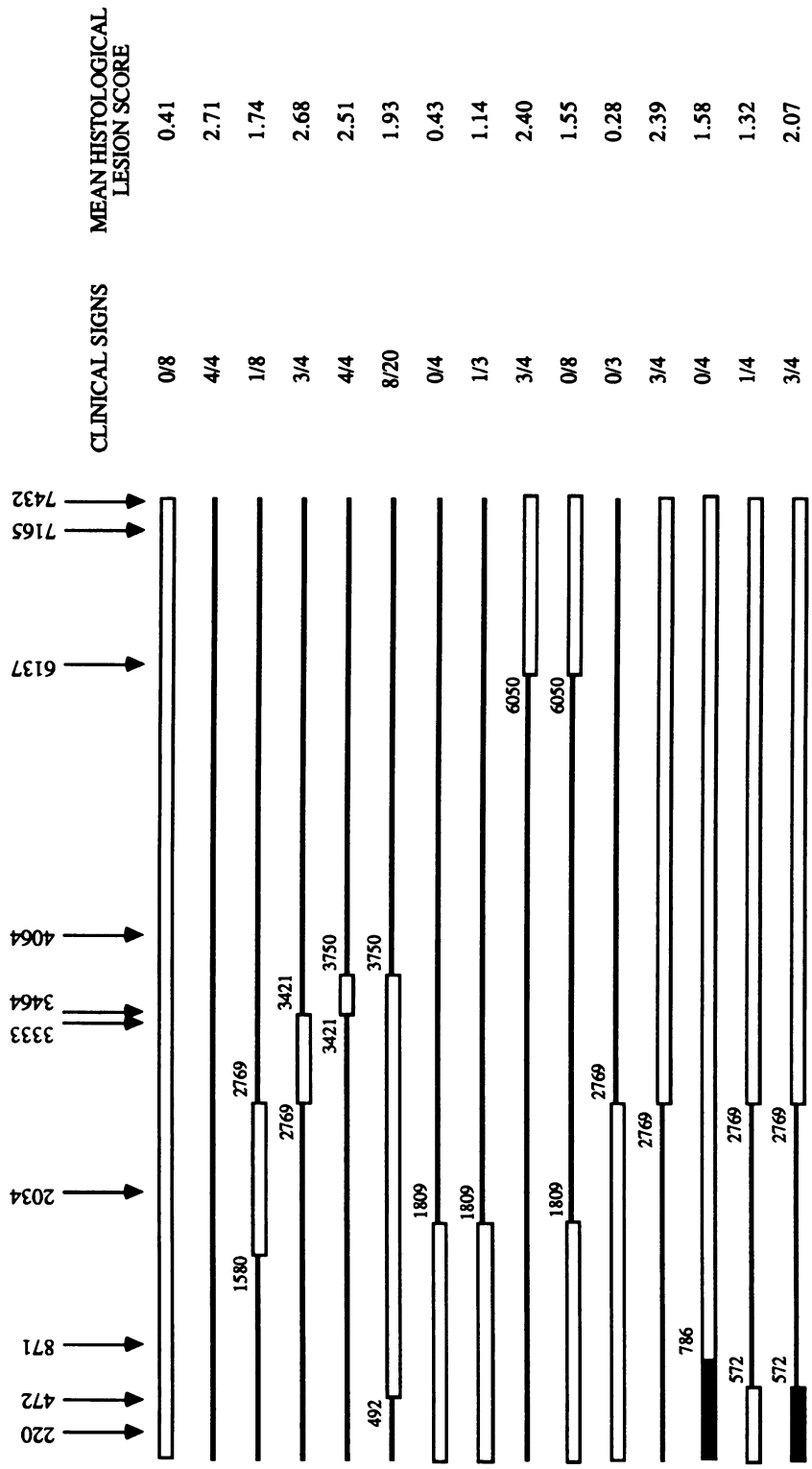

FIG. 2. Structure of recombinant viruses between the progenitor strain P3/Leon/37 and the vaccine strain P3/Leon/12a$_1$b constructed from infectious cDNAs. Recombinants were constructed by using convenient restriction endonuclease cleavage sites falling between the mutations considered to be important to the attenuation phenotype (5). After recovery of recombinant viruses by transfection of HEp-2C cells in culture at 35°C (21), the identity of the recombinants was verified by primer extension sequencing on purified virus RNA (8) covering sites of sequence difference at both sides of each crossover point. Recovered viruses were tested for neurovirulence in animals as described previously (25, 29). Clinical signs refer to the development of obvious weakness or paralysis in one or more limbs. Single lines correspond to genome regions derived from P3/Leon/37 (26), open bars to P3/Leon/12a$_1$b (24), and solid bars to regions from P3/119 (5).

therefore contained the single base deletion at position 417 (see Materials and Methods), whereas S5′3′/L was derived from the repaired pS3/FLC. Two of the recombinants (p785119/SGDW and pL472V3/S) derive their 5′ termini from the revertant virus P3/119 (5, 27). In this region, P3/119 differs from the vaccine strain only at nucleotide 472, where it has a cytidine in place of a uridine residue and is therefore like P3/Leon/37 (5). The recovered virus 785119/SGDW is therefore identical in sequence to the vaccine strain except at base 472, and L472V3/S differs from the vaccine at positions 472 and 2034 and at the position of the silent mutation at 871.

Viruses recovered by transfection were subjected to limited RNA sequence analysis (8) to confirm their configuration and tested for neurovirulence (25, 29). Figure 2 summarizes the histological and clinical findings for the recovered parental and recombinant viruses.

Effect of amino acid substitutions. Recombinants were constructed to examine the effects, singly or in combination, of the mutations at positions 2034, 3333, and 3464, which result in changes in protein structure in VP3, VP1, and P2-A, respectively (26).

Virus SV3/L differs from P3/Leon/37 only at position 2034, where the mutation causes a change from serine to phenylalanine at amino acid 91 of the coat protein VP3. In tests involving a total of eight animals, an average histological lesion score of 1.74 was obtained. Moreover, only one animal of the eight developed clinical poliomyelitis. We therefore conclude that this mutation has a definite attenuating effect on neurovirulence.

Virus SV1/L contains only the mutation at base 3333, which causes a lysine-to-arginine substitution at amino acid 286 in the coat protein VP1. This virus produced clinical signs in three of four animals and gave a lesion score of 2.68, comparable to that of P3/Leon/37. It can therefore be concluded that this mutation does not, on its own, have a significant attenuating effect on the virus. A similar conclusion can be made concerning the mutation at 3464, which causes a threonine-to-alanine substitution at amino acid 30 in the virus protease P2-A. Virus SP2/L, which differed from P3/Leon/37 only at this position, produced clinical disease in all animals and gave a lesion score of 2.51.

It was concluded that when segregated, only the mutation at position 2034 of those affecting protein sequence had a significant attenuating effect on the neurovirulence of the virus. This result is in accord with previous predictions based on the likely effects of these amino acid substitutions on protein structure (26).

We explored the possibility that the mutations at positions 3333 and 3464 act synergistically to produce an attenuating effect. A recombinant plasmid, pSCC/L, was constructed which contained all the coding changes together. This recombinant also incorporated the silent base change at position 871. Recombinant virus SCC/L was recovered in five transfection experiments from two independently constructed cDNA clones. All batches were tested for neurovirulence and gave an average lesion score of 1.93, with 8 of 20 animals showing clinical disease. Thus, SCC/L was intermediate in neurovirulence and no more attenuated than SV3/L. If the amino acid substitutions in VP1 and P2-A (or the silent mutation at nucleotide at 871) had contributed to the attenuation phenotype, SCC/L would have been more strongly attenuated than SV3/L. Thus, the two mutations at positions 3333 and 3464 have no significant attenuating effect either singly or when present together in the genome of P3/Leon/37.

Mutations in noncoding regions of the genome. Evidence has been presented previously that the nucleotide at position 472 can have a major effect on the virulence of the vaccine virus (7). The results of neurovirulence tests on viruses S5′/L and ST/L, both of which differed from P3/Leon/37 at bases 220 and 472 (as well as at 871, which maps to VP4) were consistent with this conclusion. ST/L was constructed from pSabin/GDW and therefore also had the deletion at position 417. Clinical signs were induced in only one animal, and the histological lesion scores were low (0.43 and 1.14, respectively). The difference between the two strains is thought to be due to animal or virus stock variation or both rather than the deletion at base 417. The mutation at position 220 is considered unlikely to contribute to attenuation, based on the analysis of revertant sequences (5, 10, 12). The evidence presented above for SCC/L argues against a role for the mutation at position 871 in attenuation.

Analysis of the nucleotide sequence of the revertant strain P3/119 had suggested that the mutation at position 7432 just prior to the poly(A) tract also plays a role in attenuation (5). Recombinant S3′/L which contained this mutation was therefore constructed. This recombinant also included the three silent mutations at positions 4064, 6127, and 7165. The pool virus recovered from the transfection was not temperature sensitive (*ts*) in the replicative capacity test at 40°C (P. D. Minor, G. Duncan, D. M. A. Evans, D. I. Magrath, A. John, J. Howlett, A. Phillips, G. Westrop, K. Wareham, J. W. Almond, and J. M. Hogle, J. Gen. Virol., in press), produced clinical signs in three of four animals, and induced an average lesion score of 2.40, comparable to that induced by P3/Leon/37. This contrasts with a result published previously (28) from a plaque-purified S3′/L which was subsequently found to be an atypical variant of the virus stock. The conclusion that the mutations at positions 6127 and 7165 and the 3′ end had no effect on neurovirulence was confirmed by the results from a further recombinant, S5′3′/L. This virus derived the 5′ and 3′ regions of its genome from the vaccine strain but was otherwise like P3/Leon/37. It was found to be no more attenuated than ST/L in the clinical signs (one of four animals) or lesion score induced (1.70). Furthermore, the 3′-terminal sequences of several strains of type 3 poliovirus isolated from patients with vaccine-associated disease were examined by a primer extension sequencing method. Of five viruses available, four (P3/119, P3/122, P3/131, and P3/263) had retained the 3′-terminal structure of the vaccine strain, while only one (P3/132) had the 3′-terminal structure of P3/Leon/37. Except for P3/263, all have been confirmed as highly neurovirulent in the vaccine safety test (data not shown). Back mutation at position 7432 is therefore not a necessary condition for reversion to neurovirulence. This is consistent with the results of studies on recombination between vaccine viruses (N. Cammack, A. Phillips, G. Dunn, V. Patel, and P. D. Minor, Virology, in press). It was noteworthy that by this sequencing method, P3/119 was found to have a 3′-terminal sequence of GGAG $G(A)_n$. We have previously reported that a cDNA copy of the genome of P3/119 lacked the two 3′-terminal guanosine residues (5) as shown in Fig. 1. A second independent partial cDNA clone of P3/119 has since been found to have a single guanosine, as in P3/Leon/37 (unpublished data). Similar variation has been reported between the 3′-terminal sequences of the type 1 poliovirus P1/Mahoney (10, 20), and it is possible that this reflects either variation in the virus population or the imprecise priming of reverse transcription with an oligo(dT) primer during cloning or both. These results, together with the data derived from S5′/L and

S5′3′/L, indicate that the mutation at the 3′ end of the poliovirus genome does not play a significant role in the attenuation of the Sabin vaccine strain.

Reciprocal recombinants SLR1 and SLR2. The recombinant virus SLR2 derived the 5′ half of its genome up to position 2769 from P3/Leon/37 and the remainder from the vaccine strain. It thus contained the amino acid substitutions in VP1 and P2-A as well as all the silent mutations found in S3′/L. It produced clinical signs (three of four animals paralyzed) and lesion scores (2.39) identical to those of S3′/L. This supports the conclusion that none of the mutations in the 3′ half of the genome attenuate the virus to any significant degree.

Recombinant SLR1, the reciprocal of SLR2, derived the 5′ half of its genome up to position 2769 from the vaccine strain and therefore includes both the position 472 and 2034 attenuating mutations. After recovery at 35°C, this virus failed to induce any clinical signs and gave a very low lesion score (0.28), comparable to those of Sabin/GDW, SFLC3, and standard vaccine virus P3/Leon/12a_1b. This result indicates that the two attenuating mutations act additively and are sufficient to account for the attenuated phenotype of the Sabin type 3 vaccine. A previous report of a higher lesion score for SLR1 (28) was from a test on virus recovered from a transfection done at the semipermissive temperature of 37°C, which may have selected for ts^+ revertant virus.

Removing attenuating mutations from the genome of the vaccine strain. The above results suggest that recombinant viruses which contain the P3/Leon/37 sequence at 472, 2034, or both of these positions but are otherwise identical to the vaccine strain should give rise to virus of intermediate, intermediate, and full neurovirulence, respectively. The three appropriate recombinants were therefore constructed. 119786/SGDW, like P3/Leon/37, contained cytidine at residue 472 but was otherwise entirely vaccinelike. This virus produced no clinical signs and gave a mean lesion score similar to those of SV3/L and SCC/L, i.e., 1.58, which is consistent with the view that the only significant attenuating mutation in 119786/SGDW is that at position 2034. As 119786/SGDW was significantly less attenuated than the Sabin strain, these results confirm that residue 472 is a major attenuating mutation. The recombinant LV3/S was constructed to contain a genome which had the P3/Leon/37 sequence at position 2034 but was otherwise vaccinelike. Like 119786/SGDW, this virus was of intermediate neurovirulence, paralyzing one of four animals and having a lesion score of 1.32. The virulence was comparable to that of other constructs containing a Sabin-like base at position 472 but a Leon-like sequence at position 2034. This confirms that the mutation at position 2034 is strongly attenuating.

The recombinant L472V3/S was Sabin-like except at positions 472 and 2034 (and the silent mutation at position 871). This virus produced clinical signs in three of four animals and a lesion score of 2.07, comparable to those of SLR2 and S3′/L. This lesion score and clinical effect were slightly less than those of P3/Leon/37. If this result reflects the presence of a third weakly attenuating mutation, then its effect must be very minor and not readily detectable in viruses of intermediate or low neurovirulence.

DISCUSSION

These experiments were designed to identify the attenuating mutations in the Sabin type 3 vaccine strain which determine its attenuation phenotype. The analysis was based on the World Health Organization primate neurovirulence test which is intended to detect small differences in neurovirulence which may arise between vaccine batches produced by manufacturers under stringently controlled conditions (29). Experience has shown that when this is applied to Sabin type 3 vaccines, about 2% of the animals may be expected to show paralysis and average lesion scores will range between 0.2 and 0.9. In contrast, when virulent type 3 viruses are used, most animals rapidly show paralysis and average lesion scores are over ~2.2. The response of viruses of intermediate virulence in the World Health Organization test is less well documented.

The recombinants can be divided into three groups on the basis of their clinical effect and mean lesion score, i.e., virulent, with most or all animals paralyzed and a lesion score of greater than 2.2; intermediate, with some animals paralyzed and a lesion score between 0.9 and 2.2; and attenuated, with no animals paralyzed and a lesion score of less than 0.9.

The virulent group contains the recovered P3/Leon parent virus and recombinants SV1/L, SP2/L, and SLR2. These results indicate that the mutations in VP1 and P2-A and the remaining mutations in the region from base 2769 to the 3′ end of the genome do not have a detectable attenuating effect on neurovirulence.

With one exception, the intermediate group consists of recombinants which have either the C-to-U mutation at position 472 or the mutation at position 2034 which causes a Ser-to-Phe change in VP3, but not both. This suggests that both these mutations attenuate the virus but that neither on its own is sufficient to account for the full attenuated phenotype of the vaccine strain. The one exception to this general pattern is the recombinant S5′/L, which appeared to be fully attenuated despite lacking the VP3 mutation. However, essentially the same construct, ST/L, and the very similar S5′3′/L showed intermediate neurovirulence, as did LV3/S, which had the U at position 472 of the vaccine strain, but the VP3 sequence of P3/Leon/37. Two other viruses were fully attenuated, namely, the recovered Sabin vaccine strain and SLR1. Both of these contain the VP3 and the position 472 mutations. We conclude that the fully attenuated phenotype of S5′/L is probably an artifact generated by chance mutation during the construction or virus recovery.

The above conclusions were checked by the construction of three further recombinants, LV3/S, 119786/SGDW, and L472V3/S, which have essentially vaccinelike genomes with one or both of the attenuating mutations removed. LV3/S, which has the wild-type VP3 sequence, and 119786/SGDW, which is identical in sequence to the vaccine strain except that it contains cytidine at position 472, are both of intermediate neurovirulence as expected. However, recombinant L472V3/S, which is like P3/Leon at both 472 and VP3, gave a result at the top end of the intermediate range, suggesting that this virus is slightly attenuated. This would imply the presence of one or more additional weakly attenuating mutation located elsewhere in the genome. However, the results as a whole indicate that any additional attenuating mutation must be very weak compared with those at positions 472 and 2034 and therefore difficult to detect by the current test. We conclude that the attenuated phenotype of the poliovirus type 3 vaccine strain P3/Leon/12a_1b is the product of these two point mutations which act additively.

The molecular effects of the two attenuating mutations are the topics of further research. We have shown previously that viruses which have back mutated at position 472 are strongly selected for by growth in the human gut, although the molecular basis of this is unclear (7). There is some

evidence that the mutation at position 472 may affect the secondary structure of the RNA, and this may affect the efficiency of its translation (28). The position 472 mutation has also been shown to affect significantly the replication of a P2/Lansing recombinant in the central nervous system of mice, although growth in tissue culture is normal (12). The mutation at nucleotide 2034 which gives rise to a serine-to-phenylalanine substitution in VP3 confers a *ts* phenotype on the virus (Minor et al., in press), and this may well be the basis of its attenuating effect (9). *ts* mutations have been correlated with attenuation in numerous other viruses (1).

In light of the results presented here, reversion of the type 3 vaccine would seem to require at least two point mutations. These are the position 472 back mutation to cytidine, which probably occurs in all vaccinees within a few days of vaccination (7, 15), plus at least one further mutation to counteract the VP3 serine-to-phenylalanine substitution. This may be a direct back mutation at position 2034 or, as seems more likely from partial sequence analysis of other revertants (2; unpublished data), a suppressor mutation at another site. In light of the modest changes required for reversion to neurovirulence and the high mutability of RNA genomes, it is perhaps rather surprising that paralysis associated with vaccination with poliovirus type 3 is so infrequent.

The approach of constructing recombinants via cDNA has also been used to study the genetic basis of attenuation of the Sabin type 1 strain (11, 19). In this case, the vaccine strain differs from its neurovirulent progenitor P1/Mahoney at 57 positions, and 21 of these give rise to amino acid substitutions (16). Interestingly, a strongly attenuating mutation has been identified in the 5′ noncoding region at residue 480 (17), and this presumably attenuates by a similar mechanism as that at position 472 described above. Other attenuating mutations in the type 1 vaccine strain were scattered throughout the genome (16), and their greater abundance probably accounts for the excellent safety record of this strain (3).

The work described here is the first precise determination of the genetic basis of attenuation in a widely used human vaccine. Present effort is being directed toward gaining an understanding of the molecular effects of these mutations. Such information could improve considerably the prospects for constructing improved vaccines against poliomyelitis which might be incapable of reverting to neurovirulence and which might not require the present extensive, cumbersome, and expensive safety testing during their routine preparation.

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