

Conditional Poliovirus Mutants Made by Random Deletion Mutagenesis of Infectious cDNA

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Small deletions were introduced into DNA plasmids bearing cDNA copies of Mahoney type 1 poliovirus RNA. The procedure used was similar to that of P. Hearing and T. Shenk (*J. Mol. Biol.* 167:809-822, 1983), with modifications designed to introduce only one lesion randomly into each DNA molecule. Methods to map small deletions in either large DNA or RNA molecules were employed. Two poliovirus mutants, VP1-101 and VP1-102, were selected from mutagenized populations on the basis of their host range phenotype, showing a large reduction in the relative numbers of plaques on CV1 and HeLa cells compared with wild-type virus. The deletions borne by the mutant genomes were mapped to the region encoding the amino terminus of VP1. That these lesions were responsible for the mutant phenotypes was substantiated by reintroduction of the sequenced lesions into a wild-type poliovirus cDNA by deoxyoligonucleotide-directed mutagenesis. The deletion of nucleotides encoding amino acids 8 and 9 of VP1 was responsible for the VP1-101 phenotype; the VP1-102 defect was caused by the deletion of the sequences encoding the first four amino acids of VP1. The peptide sequence at the VP1-VP3 proteolytic cleavage site was altered from glutamine-glycine to glutamine-methionine in VP1-102; this apparently did not alter the proteolytic cleavage pattern. The biochemical defects resulting from these mutations are discussed in the accompanying report (Kirkegaard, *J. Virol.* 64:195-206, 1990).

The genetic analysis of RNA viruses has, until recently, been hampered by the lack of defined mutants. The discovery that full-length cDNA replicas of RNA viral genomes can initiate authentic viral infections either as DNA molecules (44, 55) or when transcribed into RNA *in vitro* (2) has made it possible to construct and characterize mutations in RNA genomes on the DNA level. Viral mutants whose phenotypic defects can be unambiguously traced to known mutations provide powerful tools with which to study individual genetic loci and the function of individual viral proteins. In addition, the availability of defined viral mutants enables the study of genetic processes in RNA viruses, such as recombination (22) and complementation (1, 5) between viral genomes.

Infectious cDNA clones have been constructed for other strains and serotypes of poliovirus (29, 44) and for picornaviruses such as coxsackie B3 virus (20) and hepatitis A virus (6). Infectious RNA can be transcribed directly from cDNA clones of several picornaviruses (12, 37, 57). Infectious RNA has also been made *in vitro* from cDNA clones of many other positive-strand RNA viruses, including Sindbis virus (48), black beetle virus (10), and several plant viruses (2, 3, 32). Full-length cDNA clones of Q β RNA phage and their transcripts have been shown to be infectious upon transfection into Q β 's *Escherichia coli* host (51, 55). Infectious cDNAs and RNAs have also been made from satellite RNAs of plant viruses (7, 52) and from RNA viroids (9, 35, 58). In principle, mutagenesis strategies that are useful for the study of one RNA genome will prove advantageous for others as well.

The positive-strand RNA genome of poliovirus consists of approximately 7,500 nucleotides and encodes one large open reading frame that is translated into a 220-kilodalton (kDa)

protein. This polyprotein is subsequently processed into numerous polypeptides (24, 49), including the viral capsid proteins, the viral RNA-dependent RNA polymerase, and several other proteins required for replication of the virus or for interactions with its host cell.

Many poliovirus mutants displaying interesting phenotypes, such as temperature sensitivity or drug resistance, have been identified by genetic screens or selections (8). In a few cases, the mutation or mutations responsible for the observed phenotypes have been identified by reconstructing the suspected lesion into an otherwise wild-type infectious cDNA clone and showing that the original phenotype was restored. Many of the mutations thus identified have been single-nucleotide substitutions. For example, it was shown that several combinations of two point mutations in 2C can confer resistance to guanidine (43), that a single-base deletion in the 5' noncoding region can confer dactinomycin sensitivity (46), and that a single-nucleotide change in the 5' noncoding region causes pronounced changes in the neurovirulence of type III poliovirus (14). Given the high misincorporation rate of the RNA-dependent RNA polymerases of most RNA viruses (54) and of poliovirus in particular (59), a certain amount of genetic instability of mutant viruses bearing single-nucleotide substitutions is expected. Thus, mutants bearing point mutations in RNA genomes can display high levels of genotypic reversion.

Site-directed mutagenesis techniques employed with the infectious poliovirus cDNA have included inserting oligodeoxynucleotide linkers into specific restriction sites (11, 26, 31, 50), modifying restriction sites to create specific insertions and deletions (5), and introducing single-nucleotide substitutions either with individual oligodeoxynucleotides (30, 42) or with mutagenic oligodeoxynucleotide "cassettes" (27). Although a variety of viable poliovirus mutants have been obtained from these methods, most mutated cDNA molecules that are made by site-directed mutagenesis techniques have given rise to either phenotypically wild-type or

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infectious virus. Thus, the isolation of conditional, viable mutants can be laborious.

To avoid techniques that introduce individual directed mutations, circumvent the genetic instability associated with point mutations, and retain the advantage of genetic selection for specific viral phenotypes, we introduced random small deletions into an infectious poliovirus cDNA. In principle, deletion mutations should display a negligible level of reversion to a precisely wild-type sequence. Introduced randomly, there should be enough variability in the mutagenized population to allow a large number of deletion mutants to be screened for desired phenotypes. Furthermore, deletions as small as 2 nucleotides in length can be quite efficiently mapped in a 7,500-nucleotide genome on either the DNA or RNA level. We used a strategy to generate small random deletions in double-stranded circular DNA molecules, to map the introduced lesions, and to screen for and characterize the resulting mutant RNA viruses.

MATERIALS AND METHODS

Cells and viruses. Wild-type Mahoney type 1 poliovirus used in these experiments was derived from a single plaque, resulting from transfection of the infectious viral cDNA described by Racaniello and Baltimore (44). The nomenclature for viral mutants suggested by Bernstein et al. (5) was employed. Virus preparations made from initial isolates, before the lesions were demonstrated to be responsible for the mutant phenotypes, are referred to by their final name with a lowercase *i* added.

HeLa cells were grown either in suspension in minimal essential medium, Joklik modification, supplemented with 7% horse serum (GIBCO Laboratories) or in petri dishes in Dulbecco modified Eagle medium (DME) supplemented with 10% calf serum (GIBCO Laboratories). Cos-1 cells (16) were obtained from R. Mulligan (Whitehead Institute). CV1 cells (African green monkey kidney cells) were also maintained in DME with 10% calf serum. High-titer virus stocks were prepared by infecting HeLa cells in petri dishes at 37°C for wild-type poliovirus and VP1-101 and at 32.5°C for VP1-102. Plaque assays were performed as described before (22); incubations at 37°C and 39.5°C were continued for 44 h, and those at 32.5°C were for 72 h unless otherwise indicated.

Deletion mutagenesis of infectious cDNA. pPolio, a 10-kilobase-pair (kb) plasmid used as the wild-type parent for deletion mutagenesis, was derived from pSV2polio (V. Racaniello, Columbia University) by the deletion of polyadenylation sequences originally contained within the pSV2 plasmid (38).

Only preparations of pPolio DNA that were more than 90% covalently closed were used. Solutions containing 50 µg of pPolio DNA per ml, 20 µg of ethidium bromide (Boehringer-Mannheim) per ml, 10 mM Tris-OH (pH 7.5), and 2 mM MgCl₂ were incubated for 10 min at 25°C with pancreatic DNase I (Boehringer-Mannheim) at concentrations ranging from 0.02 to 20 µg/ml. Reaction products were analyzed by agarose gel electrophoresis; DNase I conditions that yielded 80 to 90% nicked molecules were chosen for preparative digests of 100 µg of DNA. The resulting DNA molecules were singly nicked by this treatment because the rate of DNase I cleavage of a nicked molecule is much lower than cleavage of a covalently closed molecule at such high concentrations of ethidium bromide (4). The preparative DNase I reaction was stopped by the addition of EDTA to 10 mM. Nicked molecules were then separated from unreacted,

covalently closed molecules by CsCl-ethidium bromide density gradient centrifugation. Nicked DNA was removed from the gradient, and the CsCl and ethidium bromide were extracted by standard methods (33).

Purified nicked DNA was split into four samples for exonuclease reactions with T4 DNA polymerase (Bethesda Research Laboratories), each reaction mix containing a different single dNTP. One-hundred-microliter solutions containing 20 µg of nicked DNA, 0.1 mM dNTP, 70 mM Tris-OH (pH 7.5), 10 mM MgCl₂, 5 mM dithiothreitol, and 12 U of T4 DNA polymerase were incubated at 37°C for 30 min before EDTA was added to 25 mM, and the four solutions were pooled and extracted with phenol. The gapped DNA was collected by ethanol precipitation, rinsed with 70% ethanol, and redissolved in TE (10 mM Tris-OH [pH 8.0], 1 mM EDTA).

The gapped DNA was treated with mung bean nuclease in reactions containing 50 µg of DNA per ml, 50 mM sodium acetate (pH 5.0), 30 mM NaCl, and 1 mM ZnSO₄ for 30 min at 30°C. Nuclease concentrations were varied from 0.002 to 0.1 U/ml to find the optimal conditions to convert gapped but not nicked circular double-stranded DNA to linear form; 0.01 U/ml was usually the optimal concentration. Following nuclease treatment, the 10-kb linear DNA was purified by preparative agarose gel electrophoresis and extracted from the low-melting-point agarose by phenol extraction. The purified double-stranded linear DNA was circularized by T4 DNA ligase (Boehringer-Mannheim) and transformed into competent *E. coli* HB101 cells by standard methods.

The DNA plasmids borne by the resulting ampicillin-resistant bacterial colonies were analyzed either individually or in pools. Colonies that were analyzed individually were picked, and minipreparations of their plasmid DNA were analyzed to screen for those bearing apparently full-length plasmids. Larger preparations of those plasmids were made by standard methods (33). Pooled plasmid preparations were made by washing the bacterial contents of several ampicillin-containing plates (approximately 2,000 colonies) into 350 ml of LB bacterial growth medium containing ampicillin (40 µg/ml). After 30 min of incubation at 37°C with shaking, chloramphenicol was added to 12.5 µg/ml. The incubation was continued overnight, and the pooled plasmid preparation was made as for a standard plasmid preparation.

Screening for poliovirus mutants. Transfections of DNA were performed at 37°C with DEAE-dextran (53), and the plates were subsequently shifted to the appropriate incubation temperature. Four standard conditions were used: HeLa cells at 32.5 and 39.5°C and Cos-1 cells at 32.5 and 39.5°C. The virus isolate was then analyzed by plaque assay on HeLa and CV1 cells at 32.5 and 39.5°C to identify any phenotypic differences from wild-type virus.

Viral plaques resulting from transfections of pooled mutant plasmid DNAs were picked into individual tubes, 0.5 ml of phosphate-buffered saline (PBS) was added, and the virus was released by freezing and thawing. Several hundred plaques were picked for each of the four conditions and screened for any deviation from wild-type phenotype by the dilution and time dependence of the complete lysis of individual monolayers in 96-well microtiter dishes (Corning) containing either CV1 or HeLa cells at the two different temperatures. Suspected host range, temperature-sensitive, or cold-sensitive mutants were further plaque purified, and their phenotypes were confirmed by plaque assay. Approximately 1 in 100 viruses picked displayed some variety of mutant phenotype.

Identification of small deletions in DNA plasmids by hetero-

duplex mapping. To find a small deletion in a 10-kb DNA plasmid encoding a mutant poliovirus, 5 μ g of the mutant DNA was digested with a restriction enzyme that yielded fragments ranging in size from 100 to 1,200 base pairs. A similar digest of wild-type plasmid DNA was also prepared and labeled at either the 5' or 3' end with 32 P by standard methods (34). After phenol extraction and collection by ethanol precipitation, the mutant DNA and 1 μ g of the labeled wild-type DNA were mixed, denatured, reannealed in formamide, and collected by ethanol precipitation as described before (23). Electrophoresis to detect the retardation of heteroduplex DNA containing single-stranded loops was performed on 6 to 8% polyacrylamide gels, polymerized with a mixture of acrylamide and bisacrylamide (29:1). Electrophoresis was carried out at 10 V/cm for 12 h in Tris-borate buffer (33). Two different digests were analyzed simultaneously to ensure that the deletion would be represented on a DNA fragment of the appropriate size. Once the small deletion had been localized to within 200 to 300 base pairs, the nucleotide sequence was determined by chemical sequencing (34). The 6-nucleotide deletion in VP1-101 was identified by sequencing a uniquely end-labeled DNA fragment derived from VP1-101 DNA spanning nucleotides 2243 to 2550 in the poliovirus genome (24, 45).

Identification of small deletions in RNAs by RNase mapping. Total cytoplasmic RNA from uninfected and poliovirus-infected cells was prepared (23). Individual samples, each containing RNA from 10^6 cells, were hybridized with different 32 P-labeled RNA molecules, transcribed from the plasmids described below. Hybridization, treatment with RNases A and T₁, proteinase K digestion, phenol extraction, and display on denaturing polyacrylamide gels to map any deletions in the viral RNA were done as described before (64) with minor modifications (22).

A set of plasmids was constructed so that transcription from the SP6 promoter in each plasmid resulted in RNA molecules complementary to the positive strand of wild-type Mahoney type 1 poliovirus. These plasmids can direct the transcription of overlapping RNA molecules with negative-strand sequences from 7440 to 1, 6516 to 1, 6304 to 4886, 5823 to 2978, 4886 to 1, 4085 to 3417, 3551 to 1, 2978 to 1, 2546 to 1, 1809 to 1, 1127 to 1, and 797 to 1. The length of negative-strand RNA made was varied by digesting the template DNA molecules with different restriction enzymes; RNA molecules used as probes were usually 800 nucleotides or less in length, so that the original molecules and their digestion products could be resolved during electrophoresis on 6% polyacrylamide gels containing 8 M urea (34).

Once a deletion had been roughly mapped on the viral genome, its exact location and sequence were determined. The 12-nucleotide deletion in VP1-102 was sequenced by base-specific chemical cleavage (34) of cDNA made by reverse transcriptase-catalyzed extension of a synthetic oligodeoxynucleotide labeled with 32 P at its 5' end (47). The primer, CCGTTTCACGGACTG, was complementary to nucleotides 2534 to 2548 in the wild-type poliovirus genome and was synthesized by C. Riser (Whitehead Institute).

Reconstruction of deletions into wild-type cDNA. Oligodeoxynucleotides for site-directed deletion mutagenesis were designed to be complementary to VP1-101 and VP1-102 virion RNAs. Oligos 114C (CTGTGTTGTCAATTTCAAGCATCTGACC) and 487C (CATGCTTCAAGCATCTGTGCTAGCGCT), for VP1-101 and VP1-102, respectively, were synthesized by J. Binkley (University of Colorado).

Gapped duplex DNA molecules (36) were prepared to expose nucleotides 1 to 2978 in the poliovirus cDNA in

single-stranded regions. pGEM3 DNA plasmids (Promega Biotec) with and without nucleotides 1 to 2978 (*Sac*I to *Ava*I sites in pSV2polio) were grown in *E. coli* hosts of *dam* and *dam*⁺ genotypes, respectively. The plasmids were linearized with different restriction enzymes, denatured, and reannealed. The gapped duplex molecules were the only circular species resulting from this treatment and thus could be purified by preparative agarose gel electrophoresis.

Oligodeoxynucleotide hybridization, DNA polymerization, ligation, and transformation into a *dam*⁺ host were performed as described by Fritz (15). The resulting bacterial colonies were immobilized on nitrocellulose filters (33). Positive colonies were detected by hybridization to the labeled mutagenic oligodeoxynucleotides in the presence of tetramethylammonium chloride (61). The presence of either the VP1-101 or VP1-102 deletion in several of the resulting plasmids was confirmed by restriction mapping and sequencing.

DNA fragments bearing poliovirus cDNA nucleotides 1 to 2954 from each of the mutant plasmid types were cloned into pPolio, replacing the wild-type sequences. The resulting full-length cDNAs were named pVP1-101 and pVP1-102 and shown to be infectious by transfection and plaque assay as described above.

Analysis of virus-specific protein synthesis. Monolayers of CV1 cells in 100-mm tissue culture dishes (Corning) were infected with wild-type or mutant poliovirus at a multiplicity of infection (MOI) of 50 PFU/cell. At the indicated times postinfection, the medium was removed, the cells were washed with PBS, and 2 ml of methionine-free DME containing 100 μ Ci of [35 S]methionine (New England Nuclear Corp.) per ml was added to each plate. Incubation was continued for the indicated times, after which the medium was removed, and the cells were washed with PBS, scraped from the dishes, and collected by centrifugation at 800 \times g for 10 min. Cells from each plate were individually resuspended in 0.5 ml of a solution containing 50 mM Tris-OH (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, and 0.1% phenylmethylsulfonyl fluoride. Following incubation on ice for 15 min, samples were centrifuged for 10 min (1,200 \times g, 4°C), and the supernatants were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (29).

RESULTS

Random deletion mutagenesis of poliovirus cDNA. A mutagenesis method was used to introduce random small deletions into a 10-kb plasmid, designated pPolio, that contains a complete cDNA copy of the poliovirus RNA genome and is infectious upon transfection into mammalian cells. The plasmid pPolio contains the simian virus 40 promoter and enhancer sequences upstream from the 7.5-kb poliovirus cDNA and, in addition, sequences from pBR322 conferring ampicillin resistance and the ability to replicate in *E. coli*. The mutagenesis procedure described here is similar to that used by Hearing and Shenk (18), except that deletions were introduced randomly into the plasmid DNA and rapid mapping methods were employed. As shown schematically in Fig. 1, this random deletion mutagenesis should yield a population of circular DNA molecules in which each molecule contains one, and only one, small deletion. The randomly mutagenized DNA molecules were then screened for those encoding conditionally defective poliovirus RNA genomes.

Mutagenesis was initiated randomly (13) within the supercoiled DNA plasmids by the action of pancreatic DNase I.

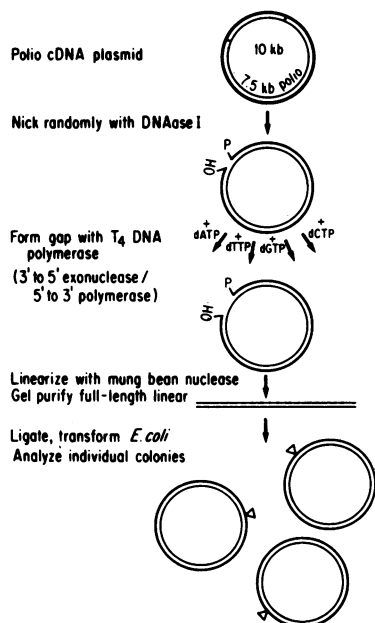


FIG. 1. Scheme for generating a population of DNA plasmids, each containing a single mutation. The triangles denote small deletions introduced into both strands of the plasmid DNA molecules. The 5' phosphate and 3' hydroxyl termini created by DNase I and T4 polymerase/exonuclease are indicated.

T4 DNA polymerase was used to convert the unique single-stranded nicks in each DNA molecule into short single-stranded gaps (Materials and Methods). Phosphodiester bonds exposed by even very short single-stranded gaps are much more sensitive to cleavage by mung bean single-strand-specific nuclease than those opposite single-stranded nicks (25); gapped molecules could thus be selectively and quantitatively converted to duplex linear molecules by mung bean nuclease. These approximately full-length linear molecules were then purified by preparative agarose gel electrophoresis, made circular by the action of T4 DNA ligase, and used to transform competent *E. coli* cells. The resulting ampicillin-resistant colonies each contained a 10-kb plasmid bearing a single small deletion. The small deletions borne by several randomly selected plasmids were mapped and sequenced (data not shown). The deletions ranged in size from 2 to 21 base pairs and were located at diverse positions throughout the plasmid sequence, as expected from the method of introduction.

These mutagenized plasmids were subsequently screened to find those encoding poliovirus genomes with conditionally expressed phenotypes. Screens for mutant viruses were performed by transfecting both individual plasmid DNA preparations and pools of mutated plasmid DNA into mammalian cells as described below.

Isolation and mapping of host range mutant VP1-101i by screening individual mutated plasmids and heteroduplex mapping of mutant cDNAs. Ampicillin-resistant colonies containing individual mutated 10-kb plasmids were picked and amplified, and the plasmid DNAs were purified. To test whether a particular plasmid encoded a poliovirus genome that was viable under any of our standard laboratory growth conditions, each DNA preparation was transfected into both HeLa and Cos-1 cells at 32.5 and 39.5°C. Any resulting viruses were plaque purified and analyzed by plaque assay for the display of conditional phenotypes such as heat or

cold sensitivity or different efficiencies of plaque formation on different cell lines. As expected from the random location and size of the introduced lesions, only 15% of the plasmids gave rise to virus under any condition, and of the individual viruses isolated, only about 10% displayed a mutant phenotype. One such mutant, isolate VP1-101i, showed a fivefold reduction in the number of plaques formed on CV1 cells relative to HeLa cells compared with wild-type virus, an effect more pronounced at 39.5°C than at lower temperatures. The plaque phenotype of this mutant will be discussed in a later section.

Small deletions can be mapped readily even when present in large plasmids by taking advantage of the altered electrophoretic mobility of DNA duplexes containing small single-stranded loops in polyacrylamide gels (19, 23, 56). For example, to determine the location of the small deletion in the plasmid DNA (designated pPolioVP1-101i) encoding viral isolate VP1-101i, heteroduplexes between pPolioVP1-101i DNA and wild-type pPolio plasmid DNA were prepared as diagramed in Fig. 2A and displayed in Fig. 2B. pPolio DNA was digested with *Hpa*II restriction enzyme, and the 3' ends of the resulting fragments were labeled with ³²P by standard methods (34). The labeled fragments were denatured and reannealed in the presence of a fivefold molar excess of unlabeled *Hpa*II-digested pPolioVP1-101i DNA. Thus, almost all of the ³²P-labeled strands were present in heteroduplex molecules following reannealing. The mobilities of almost all the labeled heteroduplex fragments remained identical with those of the homoduplex fragments. However, the heteroduplex fragments containing small single-stranded loops migrated more slowly during polyacrylamide gel electrophoresis, identifying the fragment bearing the deletion in the pPolioVP1-101i DNA. The heteroduplex fragments migrated as a doublet in lane 2 (Fig. 2B) because there were two species of heteroduplex, depending on which of the two wild-type complementary strands was present in the single-stranded loop (Fig. 2A; data not shown). As shown in Fig. 2B, the small deletion in pPolioVP1-101i DNA was localized to a 474-base-pair *Bam*HI-*Hpa*II fragment corresponding to nucleotides 2129 to 2603 in the poliovirus genome. Sequencing of this region showed that 6 nucleotides, from 2503 to 2508 inclusively, were deleted in pPolioVP1-101i DNA (termed VP1-101 in Fig. 4).

Isolation and mapping of host range mutant VP1-102i by screening pooled mutated cDNAs and RNase protection of mutant viral RNA. To screen many mutant cDNAs simultaneously, pooled preparations of mutagenized plasmid DNA, each containing DNA derived from several hundred individual mutant plasmids, were transfected into HeLa, Cos-1, and CV1 cells at both 32.5 and 39.5°C. Approximately 500 individual viral plaques, representing individual viral genomes, were picked from each of these sets of transfections and further screened by assays in microtiter dishes for heat-sensitive, cold-sensitive, and host range phenotypes. Several poliovirus mutants were isolated in this way. One such mutant, isolate VP1-102i, was found to have a host range phenotype similar to that displayed by VP1-101i, only more dramatic. Viral isolate VP1-102i showed a 13-fold reduction in the relative number of plaques formed on CV1 cells at both 32.5 and 39.5°C, and at 39.5°C the plaques formed on CV1 cells were extremely small.

Mapping deletions in mutant viruses, such as VP1-102i, isolated by this pooled-transfection protocol must be done at the RNA level, because it is clearly very difficult to determine from which DNA plasmid the genome of any particular viral isolate was derived. Small deletions in viral RNA can

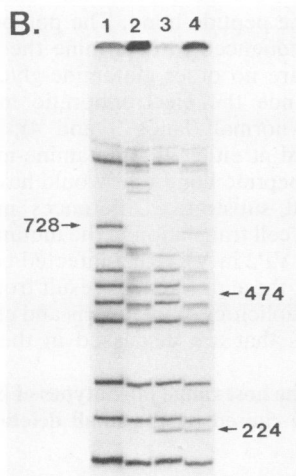
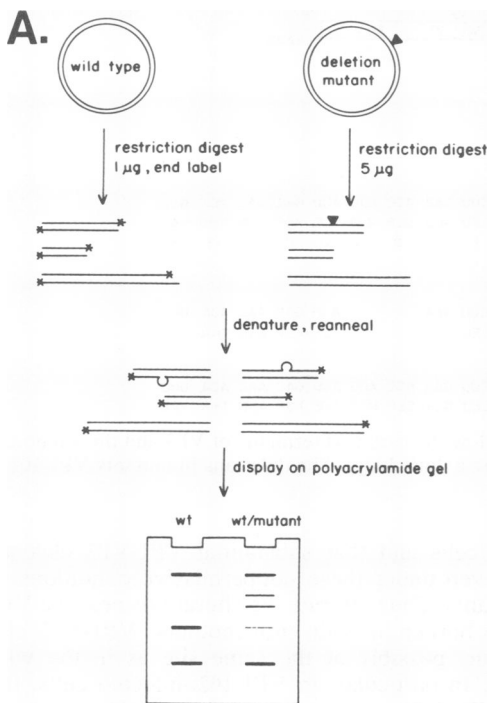


FIG. 2. (A) Diagram of protocol for mapping small deletions in DNA plasmids by using the retardation of fragments containing single-stranded loops as an assay. (B) Autoradiograph of polyacrylamide gel displaying mapping of the small deletion contained in VP1-101i. Lane 1, DNA fragments resulting from *HpaII* digest of pPolio, 3'-end labeled with ³²P, as denoted by the asterisks in panel A. Lane 2, Heteroduplexes formed by annealing *HpaII*-digested, ³²P-labeled wild-type (wt) pPolio and unlabeled *HpaII*-digested pVP1-101i DNA. Lane 3, Wild-type *HpaII* digest, as in lane 1, further digested with *BamHI*. Lane 4, Wild type-pVP1-101i heteroduplexes, as in lane 2, further digested with *BamHI*. The sizes of certain wild-type DNA fragments are indicated (in base pairs).

be readily located by using the RNase protection technique first described by Zinn et al. (64). To locate the small deletion in the viral RNA from isolate VP1-102i, total cytoplasmic RNA was prepared from mutant-poliovirus-infected cells. This RNA was hybridized with ³²P-labeled negative-sense RNA molecules transcribed in vitro from plasmids containing wild-type poliovirus cDNA sequences and promoters for SP6 RNA polymerase. Hybridization of

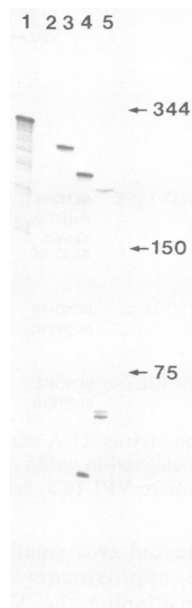


FIG. 3. Autoradiograph of a denaturing polyacrylamide gel displaying the mapping of the small deletion in VP1-102i RNA. Lane 1, Uniformly ³²P-labeled RNA containing sequences from 2292 to 2546 of the poliovirus genome as well as extraneous sequences derived from the DNA plasmid from which the RNA was transcribed by SP6 polymerase. Lane 2, ³²P-labeled RNA after hybridization to a preparation of cytoplasmic RNA from mock-infected HeLa cells and treatment with RNases A and T₁. Lane 3, ³²P-labeled RNA protected from RNase digestion by hybridization to cytoplasmic RNA from wild-type-poliovirus-infected extracts. Lane 4, RNA protected from RNase digestion by hybridization to cytoplasmic RNA from VP1-101i-infected extracts. Lane 5, RNA protected from RNases by hybridization to RNA from VP1-102i-infected extracts. The sizes (in nucleotides) and electrophoretic mobilities of single-stranded DNA fragments are marked.

the positive-sense mutant virion RNA present in the cytoplasmic extracts to the labeled complementary molecules protected the labeled negative strands from the subsequent action of single-strand-specific RNases A and T₁. The single-stranded loop formed in the labeled transcript opposite the small deletion on the mutant RNA was sensitive to RNase treatment; the altered sizes of the two resulting pieces of labeled RNA were visualized by denaturing gel electrophoresis and used to identify the location of the deletion mutation.

A representative experiment showing the mapping of the small deletion in the genomes of viral isolates VP1-101i and VP1-102i is shown in Fig. 3. Labeled DNA restriction fragments were used to determine the approximate sizes of the labeled RNA fragments following denaturing polyacrylamide gel electrophoresis. Lane 1 displays an untreated negative-strand RNA transcript approximately 320 nucleotides long, containing sequences complementary to nucleotides 2292 to 2546 of the poliovirus genome, as well as sequences transcribed from other plasmid sequences. Lane 2 shows the total disappearance of these RNA molecules upon hybridization to cytoplasmic RNA prepared from uninfected HeLa cell extracts and subsequent RNase treatment. The RNA fragment in lane 3 resulted from protection of most of the labeled probe by sequences 2292 to 2546 of the wild-type poliovirus genome and migrated consistently with its predicted size of 254 nucleotides. RNA prepared from VP1-

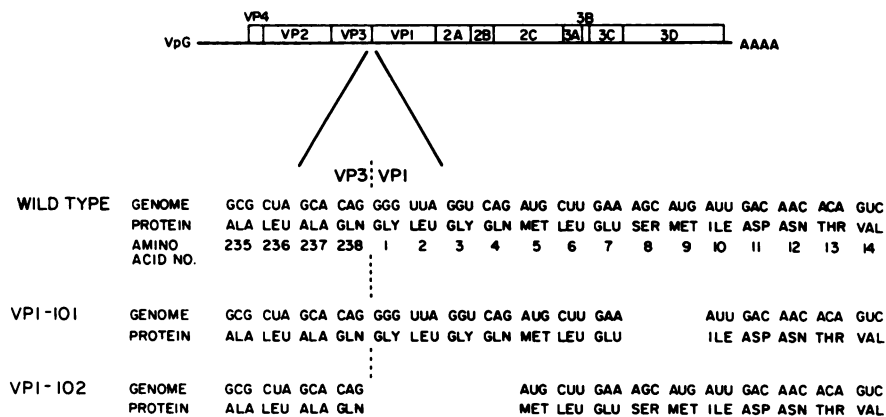


FIG. 4. Sequences of poliovirus RNA and the viral proteins in the region encoding the carboxyl terminus of VP3 and the amino terminus of VP1. The peptide bond cleaved in wild-type poliovirus infections is indicated by a dotted line. The deletions in mutants VP1-101 (isolate VP1-101i) and VP1-102 (isolate VP1-102i) are shown.

101i-infected cells protected two smaller fragments of the labeled RNA molecule, approximately 200 and 40 nucleotides long (lane 4), positioning the VP1-101i lesion near either nucleotide 2332 or nucleotide 2492 in the poliovirus genome. Similarly, the approximately 180- and 60-nucleotide fragments protected by VP1-102i RNA (lane 5) showed that the deletion was near either nucleotide 2352 or 2472 in the viral genome. The ambiguities in mapping were resolved by the sizes of protected RNA molecules with other RNA probes, and the precise lesions were then determined by sequencing (see Materials and Methods section). As discussed previously, the deletion in isolate VP1-101i spanned nucleotides 2503 to 2508. A 12-nucleotide deletion in viral isolate VP1-102i was found to be nearby and to span nucleotides 2482 to 2493. The wild-type and mutant sequences in this region of the poliovirus genome are shown in Fig. 4; the VP1-102i sequence is termed VP1-102 in the figure. These sequences encode the amino terminus of VP1, one of the viral capsid proteins.

The 12-nucleotide deletion in isolate VP1-102i is predicted to remove the four extreme amino-terminal residues of VP1. It might be expected that some aspect of proteolytic processing would be altered in this mutant, since the polypeptide bond linking the mutant VP1 and VP3 before proteolysis should be altered from the canonical glutamine-glycine (40) to a glutamine-methionine bond. Figure 5 displays the virus-specific proteins made in CV1 cells infected with wild-type and mutant poliovirus. The bands corresponding to individual capsid proteins were identified by their comigration with [³⁵S]methionine-labeled proteins from purified virions and their absence from labeled mock-infected cells (not shown). Lanes 1 and 2 show proteins of VP1-101i- and wild-type poliovirus-infected cells, respectively, labeled with [³⁵S]methionine from 3 to 4 h postinfection at 39.5°C. Lanes 3 and 4 show VP1-102i- and wild-type-infected cells labeled from 2.5 to 3.5 h postinfection at 39.5°C. The electrophoretic mobilities of both mutant VP1s differed slightly from that of wild-type VP1 in this SDS-polyacrylamide gel. The decreased mobility of VP1 in VP1-102i-infected cells was reproducible and may follow from a difference in detergent binding; the mutant VP1 is presumably slightly smaller than wild-type VP1. The altered mobility of VP1 from VP1-102i-infected cells was seen in several plaque isolates of the reconstructed mutant VP1-102 as well (see next section). It is clear, however, that the mobility of VP3 appeared to be unaltered in extracts from either VP1-101i- or VP1-102i-

infected cells and that substantial VP1-VP3 cleavage occurred even under these nonpermissive conditions for the two mutants. Thus, despite the alterations near the VP1-VP3 cleavage border in both viral mutants, VP1-VP3 cleavage can occur, possibly at the same site as in the wild-type proteins. In particular, in VP1-102i-infected cells, the predicted VP1-VP3 cleavage border was changed to a glutamine-methionine peptide bond. The polypeptide products have not been sequenced to determine the actual cleavage sites, but there are no other glutamine-glycine sites in the region. Thus, since the electrophoretic mobility of VP3 appeared to be normal (lanes 3 and 4), the proteolytic cleavage occurred at either the glutamine-methionine bond or some nearby peptide bond that would have been an even more unexpected substrate. Differences in the extent of inhibition of host cell translation in the mutant infections and in the amount of VP2 in VP1-102i-infected cells (lane 3) can be seen in Fig. 5; these differences result from differences in the effective multiplicities of infections and efficiency of viral assembly, effects that are discussed in the accompanying paper (21).

Evidence that the host range phenotypes of isolates VP1-101i and VP1-102i are caused by the small deletions. To demon-

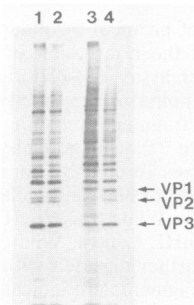


FIG. 5. Autoradiograph of an SDS-polyacrylamide gel displaying the proteins made in wild-type- and mutant-infected CV1 cells. Lane 1, Soluble proteins from wild-type-poliovirus-infected cells, labeled with [³⁵S]methionine from 3 to 4 h postinfection at 37°C. Lane 2, Proteins from VP1-101i-infected cells, labeled as in lane 1. Lane 3, Proteins from VP1-102i-infected cells, labeled from 3.5 to 4.5 h postinfection at 39.5°C. Lane 4, Proteins from wild-type-infected cells, labeled as in lane 3. The bands corresponding to wild-type VP1, VP2, and VP3 are specified.

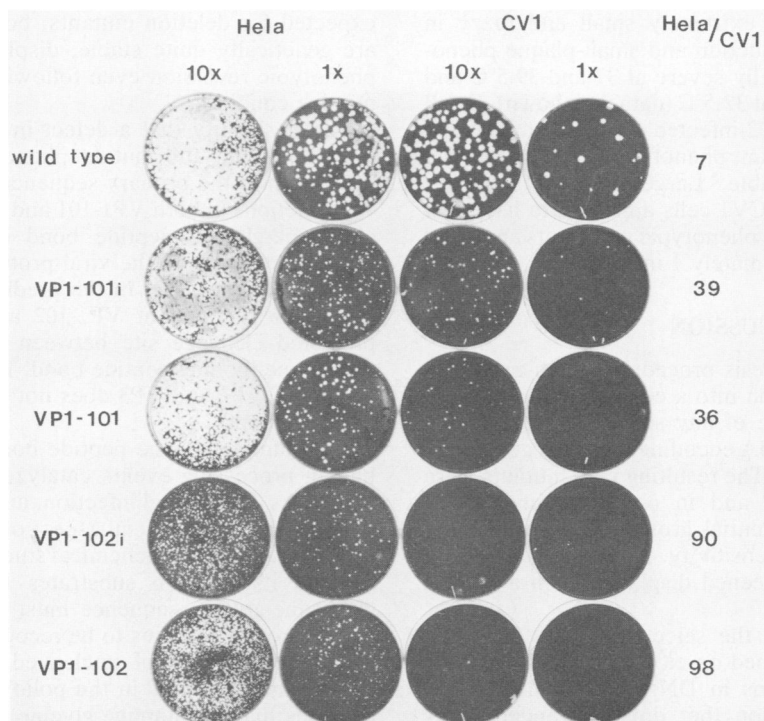


FIG. 6. Plaque phenotypes of wild-type, VP1-101i, VP1-101, VP1-102i, and VP1-102 viruses on HeLa and CV1 cells at 39.5°C. The ratios of the number of plaques formed on HeLa cells to the number formed on CV1 cells for the wild-type and mutant viruses are given in the column at right. Relative concentrations of the viral inocula are indicated above the columns of petri dishes.

strate that a mutant phenotype results from any particular mutation, it is necessary to "cross" that mutation biochemically or genetically into a wild-type genetic background and then to show that the mutant phenotype is retained. This is particularly important in the analysis of mutations in an RNA genome because the extremely high error rate of RNA-dependent RNA polymerases can lead to a high incidence of point mutations in viral stocks. The reappearance of a mutant phenotype following reconstruction of the suspected mutation into a wild-type genetic background does not, of course, strictly prove that the mutation is responsible for the mutant phenotype, a possibility that can only be ruled out by sequencing the entire genomic RNA from several plaque isolates. However, we consider the presence of a mutant phenotype in a poliovirus genome deliberately reconstructed with a candidate mutation strong evidence that the mutation is responsible for the mutant phenotype.

To determine whether the conditional phenotypes of mutant poliovirus isolates VP1-101i and VP1-102i were caused by the small deletions they bear or by some other unknown mutations elsewhere in the genome, the deletions were individually reconstructed into a DNA plasmid (pCAP) that contained only the first 2978 nucleotides of the wild-type poliovirus cDNA clone. Two DNA oligonucleotides, each 28 nucleotides in length, were designed to be complementary to the genomic strands of isolates VP1-101i and VP1-102i in the regions of VP1-encoding sequence where the mutants were known to differ from the wild type. These DNA oligonucleotides were used to introduce the specific deletions into the pCAP DNA. Plasmid DNA was prepared from those bacterial colonies that stably hybridized with the DNA oligonucleotides and thus contained the deletions. The presence of the deletions was confirmed by sequencing, and duplicates

of the newly mutant sequences were reconstructed into plasmids containing full-length poliovirus cDNA sequences.

These full-length plasmids, as well as their wild-type parent, pPolio, were transfected into HeLa cells at 32.5 and 39.5°C. Several viral plaques from each transfection were picked, and the phenotypes of the viruses therein were determined by plaque assay. The phenotypes of these viruses at 39.5°C, the condition under which the mutant phenotypes are most apparent, are shown in Fig. 6.

The DNA oligonucleotide-directed reconstruction of the deletion mutation in viral isolate VP1-101i conferred the phenotype on HeLa and CV1 cells observed in the original isolate. Thus, the location of the lesion responsible for the host range phenotype of isolate VP1-101i was substantiated, and this mutant was renamed VP1-101. At 39.5°C, VP1-101 showed a fivefold plaque reduction phenotype on CV1 cells relative to HeLa cells compared with wild-type virus. The plaques formed on both HeLa and CV1 cells were smaller than those formed by wild-type poliovirus (Fig. 6). Reduction of the number of plaques formed on CV1 cells was observed at 32.5 and 37°C as well, but the effect was less pronounced. Plaques isolated from either CV1 cells or HeLa cells infected with VP1-101, when recounted, displayed the mutant phenotype, indicating that the plaques formed under nonpermissive conditions on CV1 cells were not revertants and that the mutation was genetically stable.

Reconstruction of the 12-nucleotide deletion found in the RNA of viral isolate VP1-102i also recreated the phenotype of the original isolate (Fig. 6). Therefore, the 12-nucleotide deletion is probably responsible for both the small-plaque and the 13-fold-plaque-reduction phenotypes on CV1 cells observed in the original viral isolate, and the mutant was renamed VP1-102. Plaques formed by VP1-102 at 39.5°C,

although identifiable, were extremely small and fuzzy in appearance. The plaque reduction and small-plaque phenotypes of VP1-102 were equally severe at 37 and 39.5°C and somewhat less pronounced at 32.5°C (data not shown). Small plaques picked from VP1-102-infected CV1 cells at all temperatures displayed the mutant phenotype, showing that the mutation is genetically stable. Large plaques, however, which formed at 39.5°C on CV1 cells appeared to have lost the mutant phenotype; these phenotypic revertants appeared with a frequency of approximately 1 in 10^{-4} .

DISCUSSION

We have used a mutagenesis procedure which randomly introduces one small deletion into a covalently closed double-stranded DNA molecule of any size. The mutagenesis target was an infectious cDNA encoding the RNA genome of Mahoney type 1 poliovirus. The resulting viral mutants were screened both individually and in pools for conditional phenotypes, such as preferential growth on different host cell lines and temperature sensitivity. Approximately 1% of the plasmids or viruses screened displayed such a mutant phenotype.

The deletions carried by the selected viruses could be rapidly and accurately mapped on either the RNA or DNA level. For mapping deletions in DNA plasmids, we took advantage of the observation that double-stranded DNA fragments containing even very small single-stranded loops migrate more slowly in polyacrylamide gels than their uniformly duplex counterparts. Small single-stranded loops cause bending in duplex DNA molecules (19; J. Wuerker and K. Kirkegaard, unpublished), resulting in the observed electrophoretic retardation (17, 62). The effect is readily observable for single-stranded loops as short as 1 nucleotide (19), embedded in duplex DNA as long as 1,200 base pairs at several different positions (data not shown), and has proven useful in mapping all of the several deletions in the poliovirus cDNA thus far analyzed.

To map small deletions in large RNA molecules, we adapted the RNase protection method of Zinn et al. (64). Although it is conceivable that certain loop sequences would not be cleaved by the combination of single-stranded RNases A and T₁ used in this method, we have not encountered any. In fact, RNase protection has proven useful in mapping even certain single-nucleotide mismatches (22, 60).

The identified lesions were reconstructed into an otherwise wild-type poliovirus cDNA by oligodeoxynucleotide-directed mutagenesis. For both the mutants reported here, evidence was obtained that the observed deletions were responsible for the observed mutant phenotypes. However, when viruses are selected on the basis of conditional phenotypes, this is not always the case. We have found, for example, that spontaneous point mutations are sometimes responsible for phenotypes observed even following site-directed mutagenesis (data not shown). Thus, the precaution of testing any suspected lesion in an otherwise wild-type genetic background is necessary to show that it causes the mutant phenotype.

Two deletion mutants and their initial characterization are described here. VP1-101 and VP1-102 bear 6- and 12-nucleotide deletions, respectively, in the sequences encoding the amino terminus of the viral capsid protein VP1. Both mutants display a significant reduction in titer at 39.5°C on CV1 cells relative to HeLa cells compared with wild-type poliovirus. One of the mutants, VP1-102, displays a pronounced reduction in plaque size on CV1 cells as well. As was

expected for deletion mutants, both VP1-101 and VP1-102 are genetically quite stable, displaying very low levels of phenotypic reversion even following growth under nonpermissive conditions.

The possibility that a defect in proteolytic processing is responsible for the mutant phenotypes was suggested by inspection of the primary sequence changes in the mutants. The deletions in both VP1-101 and VP1-102 lie very near the glutamine-glycine peptide bond between VP1 and VP3, which is cleaved by the viral protease 3C (24). Indeed, the first four amino acids in the predicted protein sequence of VP1 from the mutant VP1-102 are deleted, changing the predicted cleavage site between VP1 and VP3 to a glutamine-methionine peptide bond. However, proteolytic resolution of VP1 and VP3 does not appear to be disturbed in either mutant.

A glutamine-glycine peptide bond is the target of all the known processing events catalyzed by the 3C protease of poliovirus during viral infection, and it has been thought that this sequence is a crucial aspect of substrate recognition by this protease (40). Biochemical studies of poliovirus protease 3C and its *in vitro* substrates have suggested that the glutamine-glycine sequence must be presented in properly folded protein domains to be recognized by the 3C protease (63). The presence of uncleaved as well as cleaved glutamine-glycine bonds in the poliovirus polyprotein (24, 45) confirms that a glutamine-glycine sequence is not sufficient for substrate recognition. *In vitro* studies of the action of the encephalomyocarditis virus 3C protease on the VP3-VP1 cleavage site in its polyprotein showed that the encephalomyocarditis virus 3C protease can cleave either glutamine-glycine or glutamine-alanine at this position (41). In another picornavirus, hepatitis A virus, the VP1-VP3 cleavage site is glutamine-methionine (39), as it may be in VP1-102. Here, we suggest from our *in vivo* observations that the glutamine-glycine sequence, in addition to being insufficient for recognition by the poliovirus 3C protease, is also not necessary.

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LITERATURE CITED

1. Agut, H., K. M. Kean, O. Fichot, J. Moraso, J. B. Flanagan, and M. Girard. 1989. A point mutation in the poliovirus polymerase gene determines a complementable temperature-sensitive defect of RNA replication. *Virology* **168**:302-311.
2. Ahlquist, P., R. French, M. Janda, and L. S. Loesch-Fries. 1984. Multicomponent RNA plant virus infection derived from cloned viral cDNA. *Proc. Natl. Acad. Sci. USA* **81**:7066-7070.
3. Allison, R. F., M. Janda, and P. Ahlquist. 1988. Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *J. Virol.* **62**:3581-3588.
4. Barzilai, R. 1973. SV40 DNA: quantitative conversion of closed circular to open circular form by an ethidium bromide-restricted endonuclease. *J. Mol. Biol.* **74**:739-742.
5. Bernstein, H. D., P. Sarnow, and D. Baltimore. 1986. Genetic complementation among poliovirus mutants derived from an infectious cDNA clone. *J. Virol.* **60**:1040-1049.

6. Cohen, J. I., J. R. Ticehurst, S. M. Feinstone, B. Rosenblum, and R. H. Purcell. 1987. Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J. Virol.* **61**:3035-3039.
7. Collmer, C. W., and J. M. Kaper. 1986. Infectious RNA transcripts from cloned cDNAs of cucumber mosaic viral satellites. *Biochem. Biophys. Res. Commun.* **135**:290-296.
8. Cooper, P. D. 1977. Genetics of picornaviruses, p. 133-168. In H. Fraenkel-Conrad and R. R. Wagner (ed.), *Comprehensive virology*, vol. 9. Plenum Publishing Co., New York.
9. Cress, D. E., M. C. Kiefer, and R. A. Owens. 1983. Construction of infectious potato spindle tuber viroid cDNA clones. *Nucleic Acids Res.* **11**:6821-6835.
10. Dasmahapatra, B., R. Dasgupta, K. Saunders, B. Selling, T. Gallagher, and P. Kaesberg. 1986. Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. *Proc. Natl. Acad. Sci. USA* **83**:63-66.
11. Dewalt, P. G., and B. L. Semler. 1987. Site-directed mutagenesis of proteinase 3C results in a poliovirus deficient in synthesis of viral RNA polymerase. *J. Virol.* **61**:2162-2170.
12. Duechler, M., T. Skern, D. Blaas, B. Berger, W. Sommergruber, and E. Kuechler. 1989. Human rhinovirus serotype 2: *in vitro* synthesis of an infectious RNA. *Virology* **168**:159-161.
13. Ehrlich, S. D., U. Bertazzoni, and G. Bernardi. 1973. The specificity of pancreatic deoxyribonuclease. *Eur. J. Biochem.* **40**:143-147.
14. Evans, D. M. A., G. Dunn, P. D. Minor, G. C. Schild, A. J. Cann, G. Stanway, F. W. Almond, D. Currey, and J. V. J. Maizel. 1985. Increased neurovirulence associated with a single nucleotide change in a noncoding region of the Sabin type 3 poliovaccine genome. *Nature (London)* **314**:548-550.
15. Fritz, H. 1985. The oligonucleotide-directed construction of mutations in recombinant filamentous phage, p. 151-171. In D. M. Glover (ed.), *DNA cloning: a practical approach*. IRL Press, Oxford.
16. Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**:175-184.
17. Hagerman, P. J. 1984. Evidence for the existence of stable curvature of DNA in solution. *Proc. Natl. Acad. Sci. USA* **81**:4632-4636.
18. Hearing, P., and T. Shenk. 1983. Functional analysis of the nucleotide sequence surrounding the cap site for adenovirus type 5 region E1A messenger RNAs. *J. Mol. Biol.* **167**:809-822.
19. Hsieh, C. H., and J. D. Griffith. 1989. Deletions of bases in one strand of duplex DNA, in contrast to single-base mismatches, produce highly kinked molecules: possible relevance to the folding of single-stranded nucleic acids. *Proc. Natl. Acad. Sci. USA* **86**:4833-4837.
20. Kandolf, R., and P. H. Hofschneider. 1985. Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. *Proc. Natl. Acad. Sci. USA* **82**:4818-4822.
21. Kirkegaard, K. 1990. Mutations in VP1 of poliovirus specifically affect both encapsidation and release of viral RNA. *J. Virol.* **64**:195-206.
22. Kirkegaard, K., and D. Baltimore. 1986. The mechanism of RNA recombination in poliovirus. *Cell* **47**:433-443.
23. Kirkegaard, K., and J. C. Wang. 1985. Bacterial DNA topoisomerase I can relax positively supercoiled DNA containing a single-stranded loop. *J. Mol. Biol.* **185**:625-637.
24. Kitamura, N., B. Semler, P. Rothberg, G. Larsen, C. Adler, A. Dorner, E. Emini, R. Hanecak, J. Lee, S. van der Werf, C. Anderson, and E. Wimmer. 1981. Primary structure, gene organisation, and polypeptide expression of poliovirus RNA. *Nature (London)* **291**:547-553.
25. Kroeker, W. D., and D. Kowalski. 1978. Gene-sized pieces produced by digestion of linear duplex DNA with mung bean nuclease. *Biochemistry* **17**:3236-3243.
26. Kuge, S., and A. Nomoto. 1987. Construction of viable deletion and insertion mutants of the Sabin strain of type 1 poliovirus: function of the 5' noncoding sequence in viral replication. *J. Virol.* **61**:1478-1487.
27. Kuhn, R. J., H. Tada, M. F. Ypma-Wong, J. J. Dunn, B. L. Semler, and E. Wimmer. 1988. Construction of a "mutagenesis cartridge" for poliovirus genome-linked viral protein: isolation and characterization of viable and nonviable mutants. *Proc. Natl. Acad. Sci. USA* **85**:519-523.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
29. La Monica, N., C. Meriam, and V. R. Racaniello. 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. *J. Virol.* **57**:515-525.
30. Lee, C., and E. Wimmer. 1988. Proteolytic processing of poliovirus polyprotein: elimination of 2A-mediated, alternative cleavage of polypeptide 3CD by *in vitro* mutagenesis. *Virology* **166**:405-414.
31. Li, J.-P., and D. Baltimore. 1988. Isolation of poliovirus 2C mutants defective in viral RNA synthesis. *J. Virol.* **62**:4016-4021.
32. Loesch-Fries, L. S., N. P. Jarvis, K. J. Krahn, S. E. Nelson, and T. C. Hall. 1985. Expression of alfalfa mosaic virus RNA 4 cDNA transcripts *in vitro* and *in vivo*. *Virology* **146**:177-187.
33. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
35. Meshi, T., M. Ishikawa, T. Ohno, Y. Okada, T. Sano, I. Ueda, and E. Shikata. 1984. Double-stranded cDNAs of hop stunt viroid are infectious. *J. Biochem.* **95**:1521-1524.
36. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:20-78.
37. Mizutani, S., and R. Colonno. 1985. *In vitro* synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. *J. Virol.* **56**:628-632.
38. Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**:2072-2076.
39. Najarian, R., D. Caput, W. Gee, S. J. Potter, A. Renard, J. Merryweather, G. Van Nest, and D. Dina. 1985. Primary structure and gene organization of hepatitis A virus. *Proc. Natl. Acad. Sci. USA* **82**:2627-2631.
40. Nicklin, M. J. H., H. Toyoda, M. G. Murray, and E. Wimmer. 1986. Proteolytic processing in the replication of polio and related viruses. *Biotechnology* **4**:33-42.
41. Parks, G. D., and A. C. Palmenberg. 1987. Site-specific mutations at a picornavirus VP3/VP1 cleavage site disrupt *in vitro* processing and assembly of capsid precursors. *J. Virol.* **61**:3680-3687.
42. Pelletier, J., M. E. Flynn, G. Kaplan, V. R. Racaniello, and N. Sonenberg. 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. *J. Virol.* **62**:4486-4492.
43. Pincus, S. E., and E. Wimmer. 1986. Production of guanidine-resistant and -dependent poliovirus mutants from cloned cDNA: mutations in polypeptide 2C are directly responsible for altered guanidine sensitivity. *J. Virol.* **60**:793-796.
44. Racaniello, V. R., and D. Baltimore. 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science* **214**:916-919.
45. 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. USA* **78**:4887-4891.
46. Racaniello, V. R., and C. Meriam. 1986. Poliovirus temperature-sensitive mutant containing a single nucleotide deletion in the 5' noncoding region of the viral RNA. *Virology* **155**:498-507.
47. Reilly, E. B., R. M. Reilly, R. M. Breyer, R. T. Sauer, and H. N. Eisen. 1984. Amino acid and nucleotide sequences of variable regions of mouse immunoglobulin light chains of the λ 3-subtype. *J. Immunol.* **133**:471-475.
48. Rice, C. M., R. Levis, J. H. Strauss, and H. V. Huang. 1987. Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temper-

- ature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *J. Virol.* **61**:3809–3819.
49. Rueckert, R. R., and E. Wimmer. 1984. Systematic nomenclature of picornavirus proteins. *J. Virol.* **50**:957–959.
 50. Sarnow, P., H. D. Bernstein, and D. Baltimore. 1986. A poliovirus temperature-sensitive RNA synthesis mutant located in a noncoding region of the genome. *Proc. Natl. Acad. Sci. USA* **83**:571–575.
 51. Shaklee, P. N., J. J. Miglietta, A. C. Palmenberg, and P. Kaesberg. 1988. Infectious positive- and negative-strand transcript RNAs from bacteriophage Q β cDNA clones. *Virology* **163**:209–213.
 52. Simon, A. E., and S. H. Howell. 1987. Synthesis *in vitro* of infectious RNA copies of the virulent satellite of turnip crinkle virus. *Virology* **156**:146–152.
 53. Sompayrac, L. M., and K. J. Danna. 1981. Efficient infection of monkey cells with DNA of simian virus 40. *Proc. Natl. Acad. Sci. USA* **78**:7575–7578.
 54. Steinhauer, D. A., and J. J. Holland. 1987. Rapid evolution of RNA viruses. *Annu. Rev. Microbiol.* **41**:409–433.
 55. Taniguchi, T., M. Palmieri, and C. Weissmann. 1978. Q β DNA-containing hybrid plasmids giving rise to Q β phage formation in the bacterial host. *Nature (London)* **274**:223–228.
 56. Thomas, D. C., T. A. Kunkel, N. J. Casna, J. P. Ford, and A. Sancar. 1986. Activities and incision patterns of ABC excinuclease on modified DNA containing single-base mismatches and extrahelical bases. *J. Biol. Chem.* **261**:14496–14505.
 57. van der Werf, S., J. Bradley, E. Wimmer, F. Studier, and J. Dunn. 1986. Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:2330–2334.
 58. Visvader, J. E., A. C. Forster, and R. H. Symons. 1985. Infectivity and *in vitro* mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors. *Nucleic Acids Res.* **13**:5843–5857.
 59. Ward, C. D., M. A. M. Stokes, and J. B. Flanagan. 1988. Direct measurement of the poliovirus RNA polymerase error frequency *in vitro*. *J. Virol.* **62**:558–562.
 60. Winter, E., F. Yamamoto, C. Almoguera, and M. Peruchó. 1985. A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant *c-Ki-ras* allele in human tumor cells. *Proc. Natl. Acad. Sci. USA* **82**:7575–7579.
 61. Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. Lawn. 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. *Proc. Natl. Acad. Sci. USA* **82**:1585–1588.
 62. Wu, H., and D. M. Crothers. 1984. The locus of sequence-directed and protein-induced DNA bending. *Nature (London)* **308**:509–513.
 63. Ypma-Wong, M. F., D. J. Filman, J. M. Hogle, and B. L. Semler. 1988. Structural domains of the poliovirus polyprotein are major determinants for proteolytic cleavage at Gln-Gly pairs. *J. Biol. Chem.* **263**:17846–17856.
 64. Zinn, K., D. DiMaio, and T. Maniatis. 1983. Identification of two distinct regulatory regions adjacent to the human β -interferon gene. *Cell* **34**:865–879.