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trans Rescue of a Mutant Poliovirus RNA Polymerase Function

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A series of three-nucleotide insertions was engineered into the P2 and P3 coding regions of the T7 expression plasmid pT7(τ)-PV1, which encodes a full-length copy of poliovirus type 1 (Mahoney) cDNA. When RNA derived in vitro from these mutated templates was used to transfect HeLa cells, viable virus mutants were recovered. One mutant, Sel-3D-18, which contained a single amino acid insertion in the $3D^{Pol}$ coding region, was temperature sensitive for growth at 39°C and showed defects in both RNA synthesis and P1 protein processing at the nonpermissive temperature. The RNA replication defect in Sel-3D-18 was identified at the level of RNA chain elongation. A highly specific and sensitive method was developed for analyzing the ability of mutant RNA templates to replicate in the presence or absence of helper functions provided in *trans*. This approach was used to demonstrate that RNA synthesis in Sel-3D-18 can be rescued by helper functions provided in *trans*.

Replication of picornavirus RNA has been studied in vitro by using soluble systems of crude or purified proteins and template RNA (33). One drawback of using such soluble systems, however, is that no complete system yet exists which can faithfully carry out the multiple steps involved in synthesizing authentic minus strands from input genomic plus strands and using the minus strands as templates for the generation of more plus strands. We therefore sought to address the problem of poliovirus RNA replication in vivo, using a molecular genetic complementation approach. Our initial (unpublished) experiments indicated that although RNA templates with deletion mutations in the P1 (structural protein) coding region were self-replicating, RNAs bearing deletion mutations in the P2 and P3 regions (nonstructural proteins) were neither self-replicating nor complementable in *trans*. Bernstein et al. (3), Sarnow et al. (37), and Li and Baltimore (27) used plaque assays to measure in vivo complementation efficiency between various mutant viruses bearing lesions in the 2A, 2B, 2C, 3A, or 3D coding region or in the 3′ noncoding region (3′NCR). Their data suggested that mutations in 2A, 2C, and 3A were complementable, but no rescue of the 2B, 3D, or 3′NCR mutants was detected. In addition, Dewalt and Semler (11, 12) were able to demonstrate that a 3C mutant defective in RNA synthesis was *trans* rescuable.

It was of particular interest that the data of Bernstein et al. (3) suggested that a lesion in protein 3D could not be complemented, since 3D has been identified as the viral RNA polymerase and shown to be necessary and sufficient for chain elongation (14, 15, 42). Evidence suggesting that 3D plays a role in initiation of RNA synthesis has also been presented (10, 41). Further, 3D is involved in proteolytic processing in the form of precursor polypeptide 3CD. Unlike the processing of nonstructural protein precursors from the P2 and P3 regions of the viral genome, a reaction which is efficiently mediated by the viral proteinase 3C, complete processing of the P1 substrate is mediated by 3CD, which contains both the 3C proteinase catalytic domain and a 3D domain. The 3D sequences probably assist in P1 substrate recognition and binding (22, 43).

Kuge et al. (24) and Hagino-Yamagishi and Nomoto (16) provided further intriguing evidence for the existence of *cis*-acting viral functions by showing that (i) naturally occurring defective interfering particles do not contain deletions in regions outside the capsid coding region (nucleotides 1307 to 2630), (ii) all naturally occurring defective interfering particle deletions preserved the original polyprotein reading frame, and (iii) artifically disrupting the reading frame of a capsid deletion mutant by inserting 8 nucleotides (nt) just downstream of the deletion produced a nonreplicating template which could not be rescued by helper virus. A 12-nt in-frame insertion at the same site yielded a self-replicating template. In addition, Kaplan and Racaniello (23) showed that it was possible to make in-frame deletions of almost the entire P1 region, from the translation start site at nt 747 of their clone to nt 3064, just 321 nt upstream of the P1/P2 junction, and still yield self-replicating RNA, indicating that *cis* dependency mapped to a site downstream of nt 3064.

In this report we describe a temperature-sensitive poliovirus polymerase mutant, Sel-3D-18, which is defective in both RNA synthesis and P1 protein processing. We show that the protein processing defect does not indirectly cause the defect in RNA synthesis, but rather that the defect in RNA synthesis is the direct result of the mutation in 3D which produces a polymerase whose elongation activity is defective. Finally, we show that RNA replication in Sel-3D-18 can be rescued in *trans*. The implications of these results with respect to previous observations of *cis*-dependent determinants in RNA replication are discussed.

MATERIALS AND METHODS

Construction of mutant plasmids. Construction of *Hin*fI fill-in mutations in the poliovirus subgenomic cDNA clone pMV7-2.9 has been described elsewhere (39). pMV7-2.9 contains, in a pBR322 background, a 2.9-kb segment of poliovirus type 1 (Mahoney) [PV1(M)] cDNA extending from nt 4600 to the *Eco*RI linker (nt 7524) at the 3′ terminus of the cDNA (38).

For use in RNA transfection, segments of pMV7-2.9

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containing the *Hin*fI site modifications were cloned into the transcription plasmid pT7(τ)-PV1. Plasmid pT7(τ)-PV1 consists of a full-length copy of PV1(M) cDNA under control of the bacteriophage T7 promoter and was constructed by ligating the following five fragments: (i) vector pGEM-1 (Promega Biotec) digested with *Eco*RI and *Pvu*II to remove the T7 promoter supplied with the vector; (ii) a synthetic double-stranded oligonucleotide containing, from 5′ to 3′, a blunt end, a 6-bp artificial *Stu*I site, the 17-bp phage T7 promoter, one G · C base pair, PV1 cDNA nt 1 to 7, and a 4-base 3′ overhang (nt 8 to 11); (iii) a second synthetic double-stranded oligonucleotide containing a 3′ overhang of PV1 cDNA (nt 8 to 11) complementary to the overhang of fragment 2 followed by PV1 cDNA from nt 12 to the *Bgl*I site at nt 35, with a sticky *Bgl*I end; (iv) a 5.3-kb segment of PV1 cDNA from *Bgl*I (nt 35) to *Bgl*I (nt 5318); and (v) a 2.2-kb segment of PV1 cDNA from *Bgl*I (nt 5318) to the *Eco*RI (nt 7524) linker at the 3′ end of the poly(A) tract. Fragments 4 and 5 were obtained from the vector pEV104 described previously (38).

For bacterial expression studies, a 1.6-kb *Bgl*II (nt 5601)-to-*Avr*II (nt 7205) fragment from the pMV7-2.9:*Hin*fI-derived plasmids as well as of wild-type pMV7-2.9 was cloned into the bacterial expression vector pEXC-3D (5, 34). Expression of pEXC-3D and derivatives containing *Hin*fI mutations from the *trp* promoter results in 3CD proteins that differ from wild-type 3CD only by the presence of an additional methionine residue at the amino terminus (20) and a single amino acid insertion corresponding to the altered *Hin*fI site.

RNA transcriptions and transfections. RNA was synthesized from pT7(τ)-PV1 or its *Hin*fI derivatives in 25- to 50-μl reaction mixtures containing 40 mM Tris (pH 8), 10 mM $MgCl_2$, 10 mM NaCl, 50 mg of bovine serum albumin per ml, 4 mM spermidine, 10 mM dithiothreitol, 0.5 U of RNasin (human placental RNase inhibitor; Promega) per ml, 1 mM each ATP, CTP, GTP, and UTP, 40 ng of *Eco*RI-linearized DNA template per μl, and 0.4 U of T7 RNA polymerase (Pharmacia) per μl. Incubations were carried out at 37°C for 30 to 40 min. DNase I (RNase- and proteinase-free; Worthington Biochemicals) was then added to each reaction at a concentration of 0.25 U/μl, and the reaction mixture was incubated for an additional 10 min at 37°C. RNA was analyzed by electrophoresis of 1 to 5 μl of the reaction mixture on a 1% agarose minigel. The remainder of the reaction mixture was then used for transfection without further purification.

DEAE-dextran-mediated transfections were carried out as described previously (26), with some modifications: HeLa monolayers in 60-mm dishes at 100% confluence were rinsed twice with 2 ml of TS buffer (137 mM NaCl, 4.4 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris, 0.5 mM $MgCl_2$, 0.68 mM $CaCl_2$, adjusted to pH 7.45) and then transfected with 250 μl of transfection cocktail consisting of crude RNA transcript (0.3 to 5.0 μg), 1.0 mg of DEAE-dextran per ml, and TS buffer. Transfection cocktail was applied dropwise to the center of each monolayer and allowed to spread outward undisturbed for 30 to 40 min at room temperature. Monolayers were rinsed gently with growth medium (Dulbecco modified Eagle medium supplemented with 10% fetal calf serum) and then overlaid with the same liquid medium or with semisolid medium containing 0.45% agarose (SeaKem). Incubations were carried out at 33, 37, or 39°C, as noted in the text.

Virus stocks and plaque assays. Stocks of mutant viruses grown at 33°C were prepared by harvesting monolayers and liquid supernatants 2 to 3 days posttransfection, subjecting the harvests to five cycles of freezing and thawing, and infecting fresh HeLa monolayers, which were subsequently overlaid with semisolid medium. After 2 to 3 days, well-isolated plaques were picked. (In parallel RNA transfections of HeLa monolayers grown at 33 or 37°C but overlaid with semisolid medium, small plaques too numerous to count were seen at 33°C by 38 h posttransfection, while at 37°C no plaques were seen as late as 63 h posttransfection.) Virus was twice plaque purified and then expanded by serial passage in HeLa monolayers under liquid medium. All plaque isolations and viral passages were carried out in monolayers grown at the temperature used for the initial transfection. Plaque assays of mutant virus stocks were done in six-well plates of HeLa cells under semisolid medium. Monolayers were fixed with 10% trichloroacetic acid and stained with crystal violet (19). Stocks of Sel-3D-18 used in this study were prepared at 33°C.

Wild-type virus was derived from transfection of pT7(τ)-PV1 RNA. Stocks were prepared as described above except that all incubations were carried out at 37°C. Pseudo-wild-type virus PCV-305 was derived from cDNA transfection (21).

RNA and cDNA sequencing. RNA for sequencing and cDNA synthesis was prepared from virus-infected cells as described below. Sequencing of viral RNA in the region from nt 6393 to 7154 was performed by the annealing of single-stranded DNA oligonucleotide primers to viral RNA and then extension with reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) in the presence of deoxynucleoside triphosphates, dideoxynucleoside triphosphates, and [α-^{32}P]dATP (7, 17, 36). RNA sequencing primers S7181, LV8, and PV-3D are complementary to poliovirus plus-strand nt 7181 to 7197, 6927 to 6947, and 6697 to 6717, respectively.

Synthesis of cDNA corresponding to Sel-3D-18 viral RNA was performed as described by Dildine and Semler (13). As a primer for first-strand synthesis, a single-stranded DNA oligonucleotide complementary to plus-strand nt 7431 to 7452 was used. Second-strand synthesis was initiated from random snap-back structures and was performed with the Klenow fragment of DNA polymerase I (13). The cDNA products were then digested to completion with *Bgl*II and *Pvu*II and subcloned into the *Bgl*II (5601) and *Pvu*II (7053) sites of the vector pMV7-2.9, described above. Sequencing of the resulting plasmid DNA confirmed the presence of the predicted 3-bp insertion at nt 6756 and the absence of any other changes in the region of nt 6426 to 7059. A fragment of the reconstructed pMV7-2.9:μ18 from the *Nde*I site at nt 6426 to the *Eco*RI site at nt 7524 was then cloned into the corresponding location in the plasmid pT7(τ)-PV1. RNA derived in vitro from the reconstructed pT7(τ)-PV1:μ18 was used to transfect HeLa cells, and virus stocks were obtained, as described above.

One-step growth analysis. HeLa S3 suspension cultures at a cell density of 10^7 cells per ml were infected with either mutant or wild-type virus at a multiplicity of infection (MOI) of 20. After a 30-min room temperature adsorption period, cells were diluted to 5×10^6 cells per ml and allowed to incubate at either 33 or 39°C. Harvests (consisting of cells and medium) from the 33°C infection were taken at 2, 4.25, 6, and 8 h postinfection. Harvests from the 39°C infection were taken at 2, 3, 4.25, 5, and 7 h postinfection. Each harvest consisted of 10^6 cells. Harvests were subjected to five cycles of freezing and thawing, and titers were determined by standard plaque assay at 33 or 39°C.

Labeling of infected cells with [^{3}H]uridine. For RNA syn-

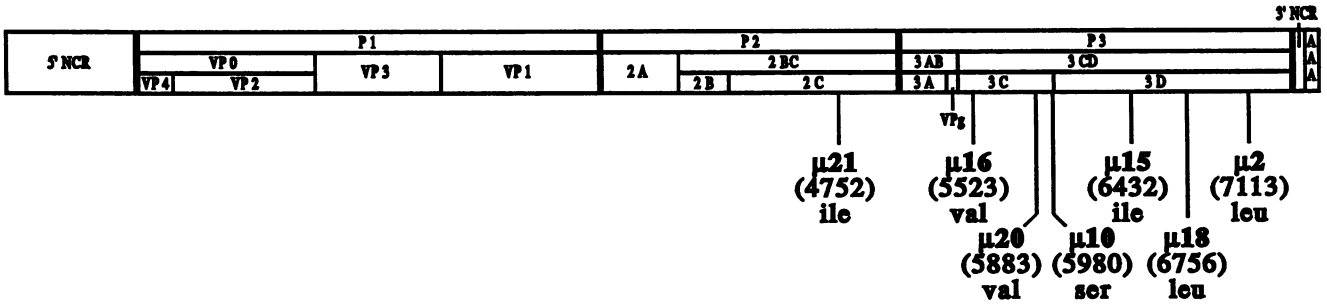

FIG. 1. Insertion mutations within the genome of PV1. Shown is a schematic representation of PV1 cDNA indicating the 5'NCR, the coding regions for polyprotein proteolytic products, the 3'NCR, and the poly(A) tract (AAA). *Hin*fI restriction sites that have been modified by in vitro mutagenesis to produce 3-bp insertions are indicated below (numbers in parentheses).

thesis comparisons, HeLa monolayers in six-well tissue culture dishes (Falcon) were infected with either mutant or wild-type virus at an MOI of 20. After a 30-min room temperature adsorption period, monolayers were fed with medium containing 5 μg of dactinomycin per ml and then incubated at either 33, 37, or 39°C. At 30 min postinfection, cultures were exposed to 10 μCi of [^{3}H]uridine per ml. Monolayers were harvested in isotonic phosphate-buffered saline (PBS) at various times postlabeling, precipitated with trichloroacetic acid, and applied to Whatman GF/C glass filters by using a vacuum device. [^{3}H]uridine incorporation was determined by scintillation counting.

For temperature shift RNA synthesis comparisons, the procedure was the same except that dactinomycin was added 5 h postinfection and [^{3}H]uridine was added at 7 h postinfection. All cultures were incubated at 33°C from 0 to 8 h postinfection and then either shifted to 39°C or kept at 33°C during the harvest period. Each time point consisted of triplicate samples.

Labeling of infected cells with [^{35}S]methionine. HeLa S3 suspension cultures were infected with either wild-type or mutant virus at an MOI of 25 and incubated for 8.75 h at 33°C at a cell density of 5×10^6/ml. Cultures were then pulsed with 12 μCi of [^{35}S]methionine per ml for 15 min at 33°C. The chase was carried out in the presence of a 100-fold excess unlabeled methionine at 33 or 39°C. Samples of 200 μl were taken at the times indicated and diluted in 1 ml of PBS. Cells were then pelleted, resuspended in Laemmli sample buffer (25), boiled, and analyzed by electrophoresis in 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) gels (25). Protein gels were then fluorographed (4) and exposed to Kodak XAR film.

Preparation of bacterial extracts and in vitro polymerase assays. Bacterial extracts were prepared from *Escherichia coli* C600 cells harboring plasmid pEXC-3D or pEXC-3Dμ6756 as described previously (5), and extracts were immediately assayed for poly(U) polymerase activity at 30°C. The amount of protein in each extract was estimated by immunoblotting several dilutions of each sample (5). Poly(U) polymerase assays were performed as described previously (18). Each extract was also assayed for primer-independent activity, which was always less than 1%.

RNA extraction and slot blot analysis. Total cytoplasmic RNA was harvested by one of two methods. RNA for sequencing and for use in cDNA synthesis was harvested by the Nonidet P-40–SDS lysis method, modified after Campos and Villarreal (6). RNA for slot blot analysis was prepared by a modification of the glutaraldehyde fixation method of Paeratakul et al. (32). Briefly, infected HeLa cell monolayers in 24-well plates (Corning) were rinsed once with PBS and then harvested and disaggregated by vigorous pipetting through a glass Pasteur pipette. Harvested cells were then slot blotted directly onto a Gene Screen Plus nylon membrane (NEN Research Products, Boston, Mass.) at a density of 4×10^4 cells per slot. Membranes were fixed in glutaraldehyde fix buffer (1% glutaraldehyde, 500 mM NaCl, 10 mM NaH_2PO_4, 40 mM Na_2HPO_4) for 60 min on ice, rinsed three times in TE8-P (100 mM Tris [pH 8], 50 mM EDTA), and then exposed to 20 μg of proteinase K per ml in TE8-P at 37°C for 30 min. Membranes were then cross-linked for 10 min on a UV transilluminator (Fotodyne) and allowed to dry.

DNA oligonucleotides for use as probes were gel purified, end labeled with [γ-^{32}P]ATP, and purified from unincorporated label by passage over a Sephadex G-50 column. Probe L391 is complementary to PV1(M) plus-strand nt 253 to 272; WB1 is complementary to PV1(M) plus-strand nt 2619 to 2638; and mu18 is complementary to Sel-3D-18 plus-strand nt 6750 to 6766, including the 3-nt fill-in at 6756.

Membranes were prehybridized for 6 h at 52°C in 1 M NaCl–1% SDS–10% dextran sulfate and then hybridized overnight to ^{32}P-end-labeled probes (10^6 cpm per 100-cm^2 filter) at 52°C in the same buffer. Hybridized membranes were washed twice at room temperature for 10 min in a buffer consisting of 0.1% SDS and 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate), twice for 30 min at 63°C in the same buffer, and twice at room temperature for 5 min in 0.1× SSC–0.1% SDS. Exposures were made on XAR film.

RESULTS

Generation of *Hin*fI fill-in mutations. Semler et al. (39) have described the construction of a series of 3-bp insertions in the P2 and P3 coding regions of PV1(M) cDNA that were used to study protein processing in vitro. Briefly, plasmid pMV7-2.9, containing nt 4600 to 7524 of PV1(M) cDNA, was subjected to partial digestion with the restriction endonuclease *Hin*fI. The 3-nt 5′ overhangs generated by the enzyme were then filled in by the Klenow reaction, and the plasmid ends were ligated. Each resulting DNA, therefore, was identical to its pMV7-2.9 parent except for the duplication of 3 nt at one of the former *Hin*fI sites at nt 4752 (designated μ21), 5523 (μ16), 5883 (μ20), 5980 (μ10), 6432 (μ15), 6756 (μ18), or 7113 (μ2). Since these 3-bp insertions fell within the coding region of the cDNA genome, they each coded for the insertion of a single amino acid at that site (Fig. 1).

For this study, each mutation was cloned into a full-length T7 RNA expression vector, pT7(τ)-PV1. This vector is

similar to the previously described pT7-1 (44) except that the distance between the T7 promoter and nt 1 of the PV1 cDNA was shortened to a single nucleotide. In vitro-synthesized RNA transcripts of pT7(τ)-PV1 mutations were used to transfect HeLa cell monolayers, and viral stocks were prepared as described in Materials and Methods. The remainder of this report concerns temperature-sensitive mutant Sel-3D-18, derived from pT7(τ)-PV1:μ18. The other mutants will be described elsewhere. Sel-3D-18 was selected for further study because the location of the lesion in the polymerase coding region corresponded to a potentially *cis*-dependent locus and because the mutant virus was markedly temperature sensitive, suggesting the existence of a temperature-sensitive polymerase function. We first sought to characterize the nature of the defect and then to assess whether it was possible to rescue the defect with helper functions provided in *trans*.

Description of Sel-3D-18. Transfection of HeLa cells with pT7(τ)-PV1:μ18-derived RNA resulted in the appearance of numerous small plaques 2 to 3 days posttransfection in monolayers overlaid with semisolid medium and grown at 33°C. No plaques were seen following RNA transfection at 37°C. Infection with Sel-3D-18 (hereafter referred to as 3D-18) resulted in the appearance of small plaques at 33°C on HeLa monolayers 3 days postinfection. At 39°C, only large plaques were detected and only at much lower dilutions (data not shown). At 37°C, a mixed-plaque phenotype was observed (data not shown). The 33°C small-plaque isolate and a 37°C large-plaque isolate were subjected to RNA sequencing in the region 6426 to 7053, surrounding the mutation site (nt 6756). The presence of the expected 3-nt insertion at the predicted site was confirmed for the 33°C isolate. The large-plaque isolate was found to contain an exact reversion and was not further characterized.

A one-step growth analysis of 3D-18 is shown in Fig. 2. At 33°C (Fig. 2A), the mutant exhibited somewhat slower initial growth kinetics than wild-type (RNA transfection-derived) virus, but by 10 h postinfection, the mutant had attained a titer only about 1 log unit lower than that of the wild type. This difference may be sufficient to account for the observed small-plaque phenotype of 3D-18. At 39°C (Fig. 2B), the temperature-sensitive defect manifested itself as a difference in titer of nearly 5 log units between mutant and wild type.

Measurement of [^{3}H]uridine incorporation in mutant- and wild-type-infected cells at 33 and 39°C (Fig. 3A and B) demonstrated that RNA synthesis of the mutant was temperature sensitive. Very little difference was observed at 33°C between the kinetics of mutant and wild-type RNA synthesis, with both incorporation curves showing peak rates of synthesis occurring between 7 and 12 h postinfection (Fig. 3A). At 39°C (Fig. 3B), the wild-type RNA synthesis rate peaked between 2 and 5 h postinfection, but almost no incorporation of label could be detected in cells infected by the mutant virus. It should be noted that the data shown in Fig. 3 were obtained from infected monolayer cultures, whereas the data of Fig. 2 were obtained from infected suspension cultures. This difference probably accounts for the discrepancy in kinetics of viral growth and RNA synthesis. When one-step growth analysis was performed in monolayers, the exponential rise in RNA synthesis was seen to coincide temporally with the increase in virus titer (data not shown).

We next examined RNA synthesis under conditions of temperature shift (Fig. 3C and D). Infected cells were incubated at the permissive temperature of 33°C for 8 h to allow some accumulation of viral RNA and protein and to bypass any temperature-sensitive defects in the early phase of the infectious cycle. At 8 h postinfection, cells were either shifted to the nonpermissive temperature of 39°C or kept at 33°C. The data showed a sharp, sustained increase in the rate of wild-type RNA synthesis (Fig. 3C) following the upshift. In 3D-18, however, the initial increase in RNA synthesis rate was not maintained, and by 30 min postshift, [^{3}H]uridine incorporation had come to a halt (Fig. 3D).

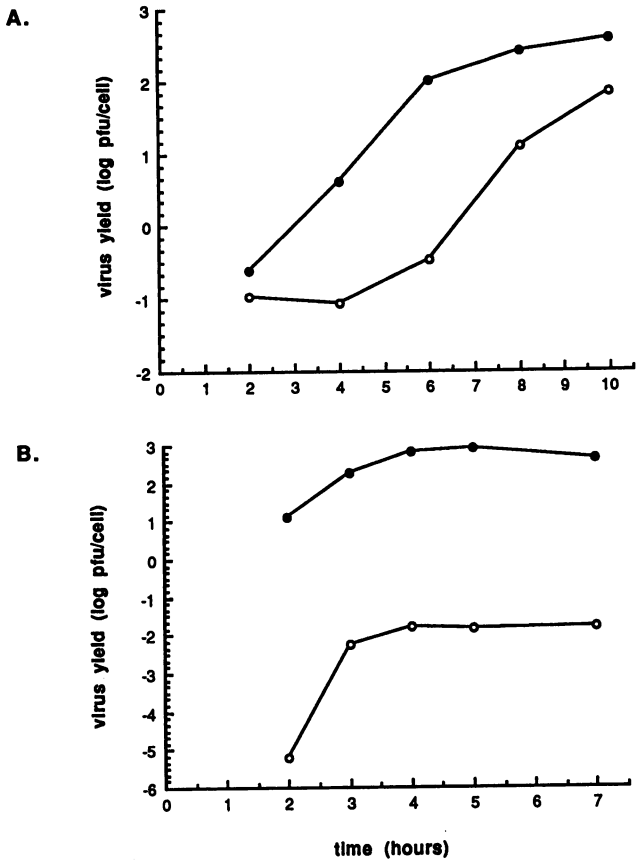

FIG. 2. One-step growth analysis of 3D-18. HeLa suspension cultures were infected with either 3D-18 (○) or wild-type [pT7(τ)-PV1 transfection-derived] virus (●) and then incubated at either 33°C (A) or 39°C (B). Cultures incubated at 33°C were harvested at 2, 4, 6, 8, and 10 h postinfection. Cultures incubated at 39°C were harvested at 2, 3, 4, 5, and 7 h postinfection. All harvests were then subjected to five cycles of freezing and thawing and were titered on HeLa cell monolayers at the same temperatures as the original harvests.

To determine whether the 3D lesion had any effect on protein synthesis or processing, a [^{35}S]methionine pulse-chase experiment was performed (Fig. 4). HeLa suspension cultures were infected with either mutant or wild-type virus and incubated for 8.75 h at 33°C to accumulate virus-specific products. Cultures were then pulsed for 15 min with [^{35}S]methionine and chased with excess unlabeled methionine at either 33 or 39°C. Figure 4A illustrates the wild-type protein processing phenotype, showing the rapid disappearance of the high-molecular-weight precursor proteins P1, P2, and P3 concurrent with the appearance of proteolytic products. Although wild-type processing at 39°C was somewhat slower than at 33°C, by 75 min after the chase very little of the P1, P2, or P3 precursor was detectable. These results are in

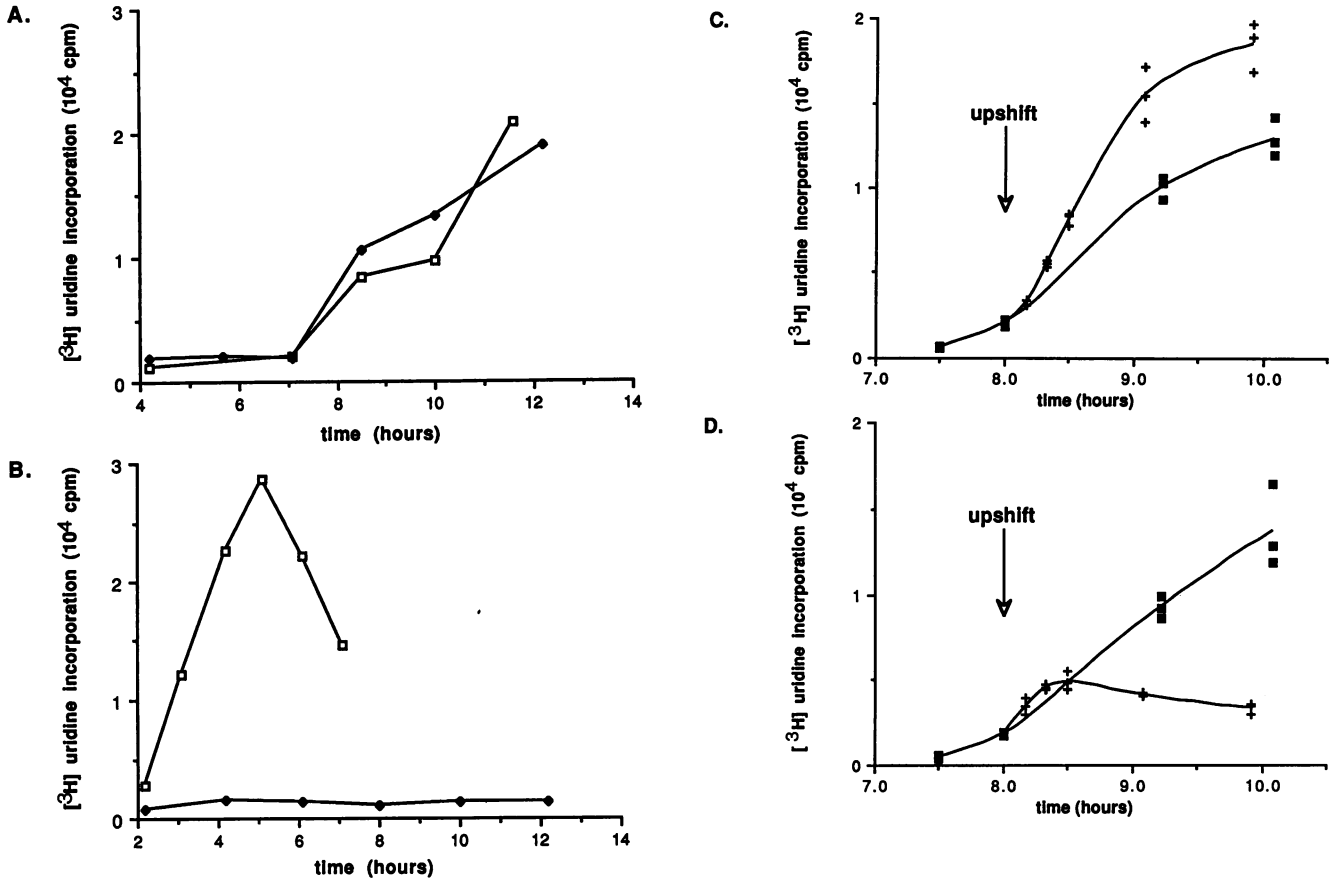

FIG. 3. Comparison of mutant and wild-type RNA synthesis. For fixed-temperature comparisons (A and B), HeLa monolayers were infected with either 3D-18 (◆) or pT7(τ)-PV1 transfection-derived wild-type virus (□) at an MOI of 20. The experiment was carried out as described in Materials and Methods. (A) [^{3}H]uridine incorporation at 33°C; (B) [^{3}H]uridine incorporation at 39°C. For temperature shift comparisons (C and D), HeLa monolayers were infected with either 3D-18 (D) or pT7(τ)-PV1 transfection-derived wild-type virus (C) and tested for [^{3}H]uridine incorporation as described above except that dactinomycin was added at 5 h postinfection and [^{3}H]uridine was added at 7 h postinfection. All cultures were incubated at 33°C until 8 h postinfection, at which time half of each culture was shifted to 39°C (+) and the other half was kept at 33°C (■). Each time point consisted of triplicate samples. Time shown is hours postinfection.

contrast with those shown in Fig. 4B, which illustrates the processing phenotype of 3D-18. Whereas the mutant processing profile at 33°C was the same as that of the wild type at 33°C, the 39°C protein processing profile of the mutant showed a substantial and specific defect in P1 processing. Figures 4C and D are densitometric plots of the autoradiograms in Fig. A and B comparing precursor-to-product ratios between mutant and wild type at 33 and 39°C.

We next addressed whether the observed defect in RNA synthesis was a direct consequence of altered polymerase function at 39°C, as suggested by the data of Fig. 3, or whether the defect in RNA synthesis was an indirect consequence of the temperature-sensitive protein processing of the P1 precursor polypeptide. To test these possibilities, a segment of pMV7-2.9:μ18 cDNA containing the *Hin*fI 6756 fill-in was cloned into the bacterial expression vector pEXC-3D (5, 35), and the resulting plasmid, pEXC-3Dμ6756, was used to transform *E. coli* C600 cells. Upon induction, pEXC-3D, which contains poliovirus 3CD sequences driven by the *trp* promoter, directs the synthesis of a protein which is identical to wild-type 3CD but for an extra amino-terminal methionine (20). Approximately 50% of the bacterially expressed 3CD is correctly processed to yield authentic 3D (5). Bacterial extracts from pEXC-3D- and pEXC-3Dμ6756-transformed cells, induced and grown at 30 or 37°C, were assayed in vitro for poly(U) polymerase activity at 30°C. [^{3}H]UMP incorporation after 60 min was about 40% lower in the mutant 30°C extracts than in wild-type 30°C extracts (Fig. 5). In 37°C extracts, however, mutant polymerase showed only about 10% of wild-type activity, a decrease of fourfold from the activity ratio in 30°C extracts. This result confirmed that the defect in 3D-18 directly affected polymerase elongation activity in a temperature-dependent fashion.

Finally, to show that the phenotype of 3D-18 was due solely to the 6756 lesion and not to a fortuitous second site change, genomic cDNA was made from 3D-18 viral RNA, and a segment corresponding to nt 6486 to 7053 was sequenced and cloned into pT7(τ)-PV. Virus derived from the reconstructed pT7(τ)-PV:*Hin*fIμ18 was then assayed for temperature sensitivity and defective protein processing. The results were in agreement with those of the original 3D-18 isolate (data not shown).

***trans* rescue of Sel-3D-18.** Having identified the functional defect of 3D-18, we were interested in determining whether RNA synthesis could be rescued in vivo at the nonpermis-

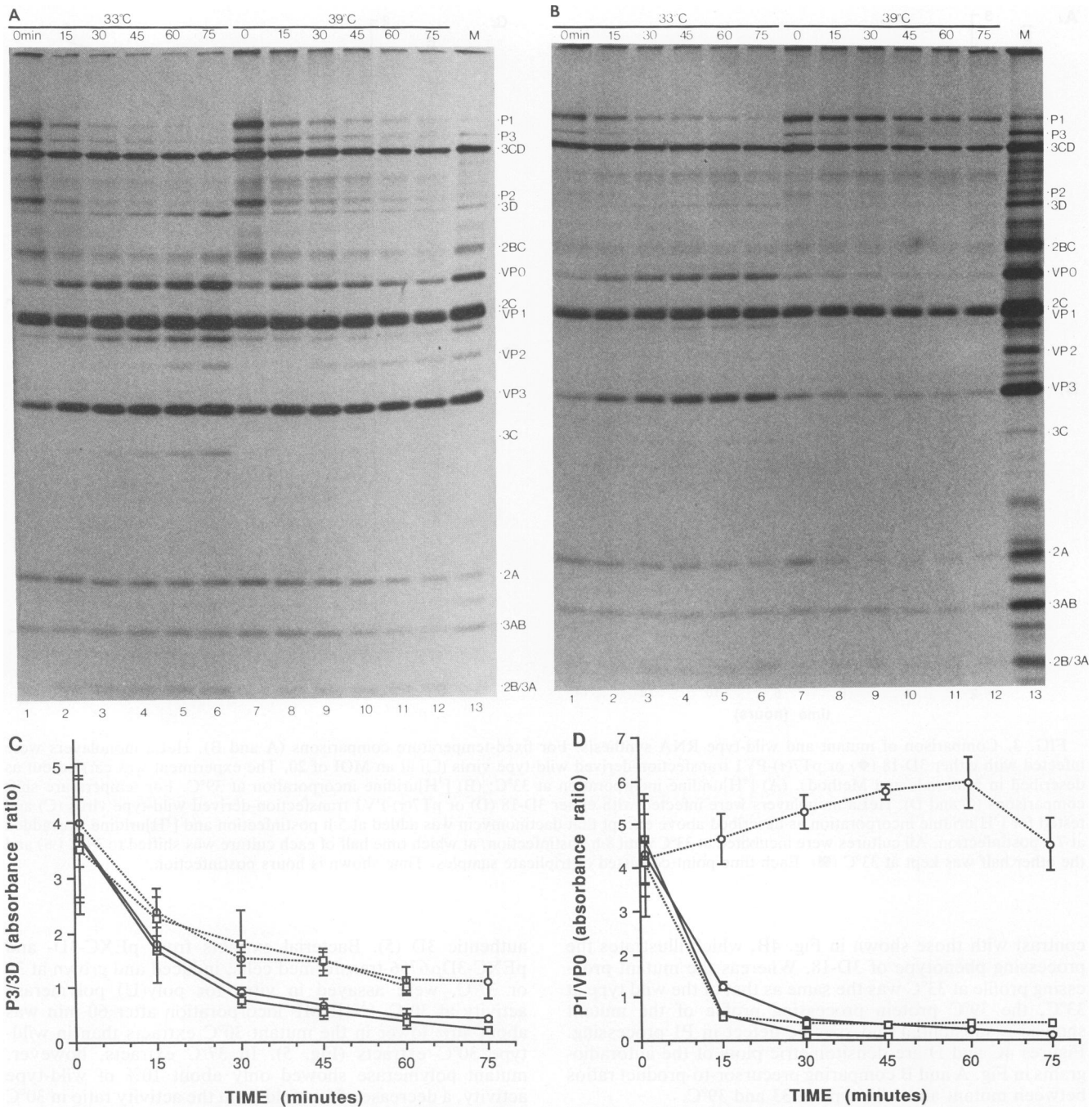

FIG. 4. Protein processing comparison of 3D-18 and wild-type poliovirus at 33 and 39°C. (A and B) HeLa S3 suspension cultures were infected with either wild-type virus (A) or 3D-18 (B) and incubated for 8.75 h at 33°C. Cultures were then pulsed with [^{35}S]methionine for 15 min at 33°C and chased with excess unlabeled methionine at 33 or 39°C. Samples were taken at 0, 15, 30, 45, 60, and 75 min postinfection, as indicated above each lane, and were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes M, wild-type poliovirus-infected cell marker. (C) Densitometric analysis of P3 protein processing. Densitometric tracing of the lanes in panels A and B was used to estimate the precursor-to-product concentration ratio of P3 to 3D in the mutant (○) or wild type (□) at 33°C (——) or 39°C (· · ·). (D) Densitometric analysis of P1 protein processing. The ratios of P1 to VPO concentration are plotted for each time point. Symbols are as for panel C. Three different exposures of each gel were scanned three times per lane, and mean areas under the peak were taken to represent the concentrations of P1, P3, VPO, and 3D. Error propagation formulas were used to calculate the standard error of the mean (error bars) for each ratio.

sive temperature by wild-type proteins acting in *trans*. As rescuing virus, we chose PCV305 (21), which is identical to PV1(M) except that the first 627 nt are derived from the homologous region of the closely related picornavirus, coxsackievirus B3. The 627-nt substitution falls entirely within the 5′NCR. All PCV305 proteins, therefore, are wild-type poliovirus proteins, and PCV305 has a wild-type poliovirus phenotype in tissue culture. The rationale for using PCV305

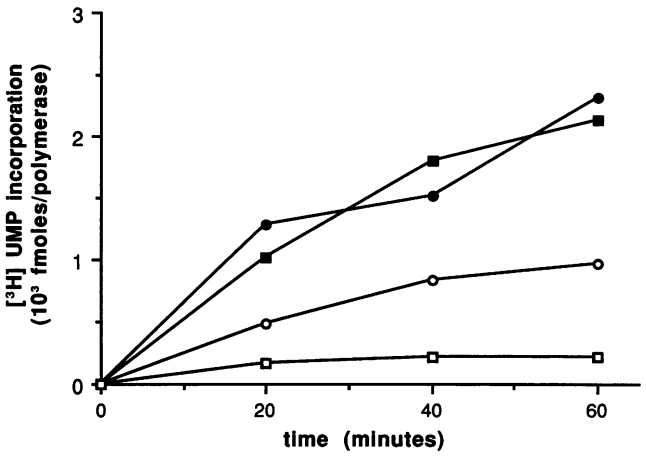

FIG. 5. Measurement of in vitro poly(U) polymerase activity. C600 bacterial cultures harboring pEXC-3D or pEXC-3Dμ6756 (see text for details) were induced and grown at 30 or 37°C. Crude extracts were prepared by sonication and assayed immediately for poly(U) polymerase activity at 30°C. The amount of poliovirus $3D^{Pol}$ in each extract was estimated by immunoblotting several dilutions of each sample, and the values for incorporation of [^{3}H]UMP were normalized for the amount of poliovirus polymerase in each extract. Bacteria expressed similar amounts of wild-type and mutant enzyme; in this experiment, both bacteria expressing wild-type enzyme and those expressing mutant enzyme produced approximately twice as much $3D^{Pol}$ at 37°C than at 30°C as a result of slight differences in growth rates and induction protocols at the two temperatures. Symbols: ●, wild-type polymerase activity in cultures grown at 30°C; ■, wild-type polymerase activity in cultures grown at 37°C; ○, mutant polymerase activity in cultures grown at 30°C; □, mutant polymerase activity in cultures grown at 37°C.

as rescuing virus was that the coxsackievirus B3 sequences in the 5'NCR could serve as a template marker, which would make it possible to use poliovirus-specific single-stranded oligonucleotide DNA probes (Fig. 6A) to distinguish between replication of the 3D-18 template, which has a poliovirus 5'NCR, from the background of PCV305 replication in coinfected cells.

The results of one such complementation experiment are shown in Fig. 6B to D. HeLa monolayers were infected with either 3D-18, PCV305, or both viruses together or were mock infected. Cells were then incubated at 33 or 37°C, with some of each culture being upshifted or downshifted after 4.5 h. All cultures were harvested at 7 h postinfection, and total cellular RNA was assayed by slot blot hybridization either to WB1, a probe specific for PV1 capsid sequences present in both viruses, or to L391, a probe specific for PV1 5'NCR sequences present only in 3D-18. 3D-18 RNA was detected by both probes at 33°C (Fig. 6B) but not at 37°C (Fig. 6C). PCV305 RNA, on the other hand, was detected at both temperatures. 3D-18 RNA could be detected in upshifted cells by probe L391 only when 3D-18 was used in coinfection with PCV305 and not when used alone (Fig. 6D). 3D-18 RNA replicates slowly at 39°C (see also Fig. 7) but faster at both 33°C (either alone or in the presence of PCV305) and at 39°C in the presence but not the absence of PCV305 helper. Thus, in the downshift experiment (Fig. 6E), no signal could be detected in RNA from cells infected with 3D-18, but a faint signal of 3D-18 RNA was detectable in cells coinfected with PCV305, presumably because of the accelerated RNA synthesis during the 39°C phase of the incubation. Coinfected cells incubated first at 33°C then at 39°C (Fig. 6D), on the other hand, showed a stronger signal for 3D-18 RNA since replication was faster at both temperatures.

To rule out RNA recombination between PCV305 and 3D-18 as a possible explanation for the apparent rescue, the complementation experiment was repeated (Fig. 7) using probe mu18, an oligonucleotide which hybridized to the region surrounding and including the 3-nt insertion in 3D-18. Under appropriate conditions of stringency, probe mu18 could be used to discriminate between mutant and wild-type templates (Fig. 7C). Figure 7A shows slot blot hybridizations of 3D-18 RNA detected by probe mu18 at the indicated times postinfection in cells incubated at either 33 or 39°C. Virus 3D-18 replicated well at 33°C in either the presence or absence of PCV305 helper. At 39°C, RNA was barely detected at 8 h postinfection in the 3D-18 infection. However, a strong signal was present at 4 h at 39°C in the coinfection with PCV305, confirming the rescue of 3D-18. Figure 7B is a control of PCV305-infected cells probed with WB1 to demonstrate the time course of RNA replication in cells infected with the helper virus at 33 and 39°C.

DISCUSSION

We constructed several poliovirus mutants whose genomes encoded single amino acid insertions at various sites in the P2 and P3 regions. We selected one of these mutants, 3D-18, for further study because of its markedly temperature-sensitive phenotype. Our characterization of 3D-18 revealed that the mutant virus grew to titers within 1 log unit of wild-type titers at the permissive temperature of 33°C but grew to titers 5 log units lower at 39°C. RNA synthesis as measured by [^{3}H]uridine incorporation was not significantly different from wild-type RNA synthesis at 33°C but was drastically curtailed at 39°C. To get a better insight into the nature of the RNA synthesis defect, we examined RNA synthesis under conditions of temperature upshift. Cells were thus allowed to accumulate RNA and protein for several hours at the permissive temperature before being abruptly switched to 39°C. We found that the mutant RNA synthesis ceased within 30 min of the upshift, although a brief increase in the rate of synthesis was seen in the first 10 min at 39°C. A sustained increase in the rate of wild-type RNA synthesis was observed at 39°C.

It had been shown previously that P1 protein processing by the viral proteinase 3C occurred inefficiently in vitro (29) but that the addition of 3D sequences in the form of a precursor such as 3CD allowed P1 processing to occur normally (22, 43). We analyzed protein processing in vivo in 3D-18 to determine whether the 3D lesion had any effect on protein processing. Indeed, we discovered that whereas at 33°C processing of all viral proteins proceeded normally in 3D-18, at 39°C a selective defect in P1 but not P2 or P3 processing was present.

An important question to be answered then was, is the RNA-synthesis defect observed in 3D-18 due to a faulty polymerase, or is it secondary to a defect in protein processing? There was good reason to believe that the protein processing defect, while undoubtedly contributing to the viral growth phenotype, had little or no effect on RNA synthesis. The evidence for this conclusion came from experiments in this laboratory (unpublished) and other laboratories (8, 9, 16, 24, 28, 30, 31) showing that deletions in the P1 capsid region resulted in subgenomic RNA templates fully capable of RNA replication. Since the lesion in 3D-18 disrupted P1 processing but not P2 or P3 region processing,

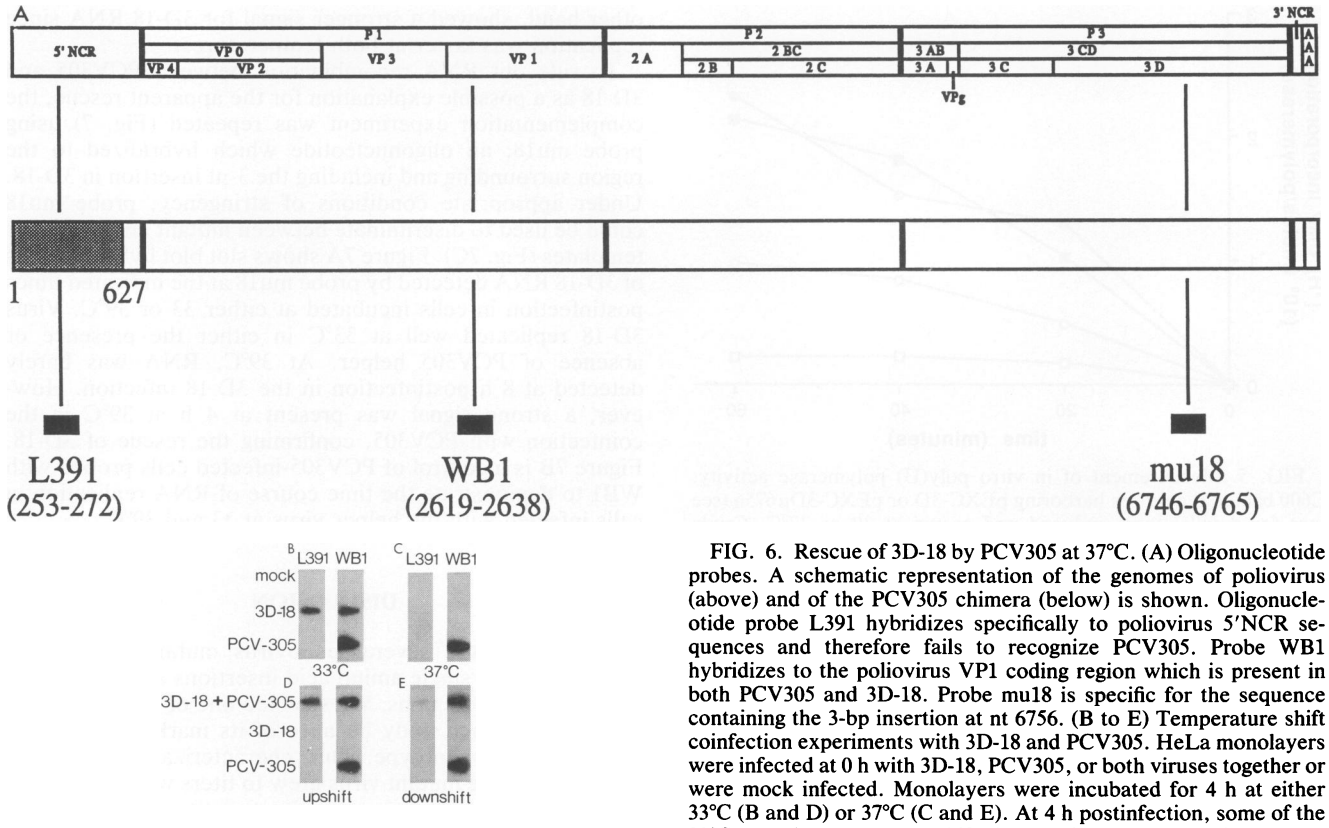

FIG. 6. Rescue of 3D-18 by PCV305 at 37°C. (A) Oligonucleotide probes. A schematic representation of the genomes of poliovirus (above) and of the PCV305 chimera (below) is shown. Oligonucleotide probe L391 hybridizes specifically to poliovirus 5'NCR sequences and therefore fails to recognize PCV305. Probe WB1 hybridizes to the poliovirus VP1 coding region which is present in both PCV305 and 3D-18. Probe mu18 is specific for the sequence containing the 3-bp insertion at nt 6756. (B to E) Temperature shift coinfection experiments with 3D-18 and PCV305. HeLa monolayers were infected at 0 h with 3D-18, PCV305, or both viruses together or were mock infected. Monolayers were incubated for 4 h at either 33°C (B and D) or 37°C (C and E). At 4 h postinfection, some of the 33°C monolayers were upshifted to 37°C (D); the remainder were kept at 33°C (B). Similarly, the 37°C monolayers were either kept at 37°C (C) or downshifted to 33°C (E) 4 h postinfection. Monolayers were harvested 7 h postinfection, and extracted cellular RNA was slot blotted and probed with ^{32}P-end-labeled oligonucleotide probe L391 or WB1. Each spot represents the hybridization signal from 4 $\times$ 10^4 cells.

it would not be expected to have a significant effect on RNA replication.

We did a further test to show that the defect in RNA synthesis existed independently of the defect in protein processing. Extracts were prepared from bacteria expressing either wild-type-derived or μ18-derived 3D under the direction of the *trp* promoter. In vitro poly(U) polymerase activity was measured in extracts prepared at either 30 or 37°C. We found that mutant polymerase activity at 37°C displayed a fourfold temperature sensitivity over the activity at 30°C, indicating that the lesion in 3D had a direct effect on the elongation activity of the polymerase in vitro. This result is consistent with the data obtained from the in vivo [^{3}H]uridine incorporation temperature shift experiment discussed above. Our interpretation is that the 3D-18 polymerase synthesized at 37 or 39°C or shifted from 33 to 39°C is misfolded and that this misfolding accounts for both the low level of in vitro elongation activity in extracts from cultures grown at 37°C and for the decline of viral RNA synthesis seen in vivo within 30 min after shifting infected cells to 39°C. It is also conceivable that 3D-18 polymerase synthesized in vivo at 33°C is stabilized by its template interactions. This would account for the transient increase in elongation rate in vivo in upshifted 3D-18-infected cultures. Alternatively, the 3D-18 polymerase could have an additional defect in initiation.

Having demonstrated that 3D-18 had a temperature-sensitive defect in polymerase elongation activity, we sought to test whether that function could be rescued in *trans*. We used a biochemical assay rather than a plaque production assay, as had been used by previous authors. Sarnow et al. (37), Bernstein et al. (3), and Li and Baltimore (27) used plaque assays to measure the degree of complementation between coinfected pairs of mutant viruses. Since each mutant had a characteristic plaque phenotype or temperature sensitivity, it had been possible to compare the yield of each virus in the coinfection with yields in single infections and thereby to quantitate the degree of complementation.

While the above experiments were an important step toward the understanding of the *cis* or *trans* activity of viral replicative functions, the plaque assay technique suffers both from low sensitivity and low specificity compared with the technique of strand-specific oligonucleotide hybridizations used in this study. For a virus to produce a plaque on a monolayer, the virus must be able to perform each step of a complete replicative cycle, including adsorption, penetration, uncoating, protein synthesis, protein processing, host shutoff, RNA replication, assembly, morphogenesis, and release. Mutants that are defective in one or more steps in this sequence may fail to produce visible plaques even though the process of interest, RNA replication, might remain intact. Furthermore, for a plaque to be grossly visible, viruses must go through several rounds of infection, release, and reinfection over the course of 2 to 3 days. During this time there is ample opportunity for the selective amplification of rare recombinants or revertants, which could cause falsely positive rescues. In addition, the evaluation of plaque phenotype is a somewhat subjective process and can therefore be prone to systematic error. Finally, two

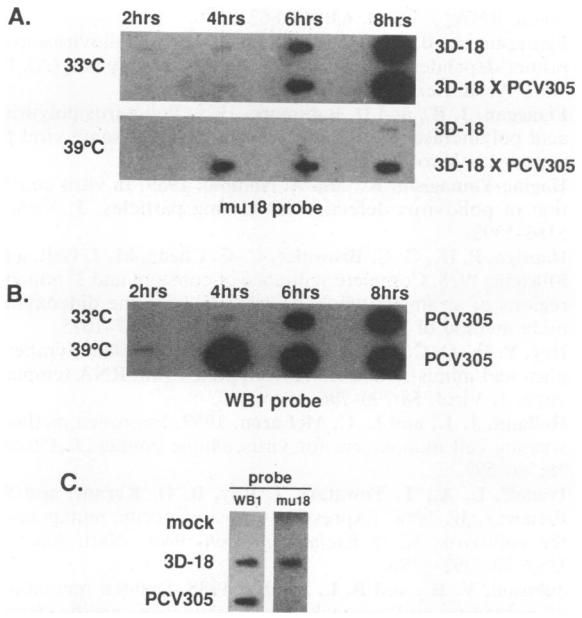

FIG. 7. PCV305 rescue of 3D-18 at 39°C: experiment to rule out recombination. HeLa cell monolayers were infected with 3D-18, PCV305, or both viruses simultaneously. After a 30-min adsorption at room temperature, cells were incubated at either 33 or 39°C for 2, 4, 6, or 8 h, harvested, and slot blotted as described in Materials and Methods. Each spot represents the hybridization signal from 4×10^4 cells. (A) Rescue of mutant 3D-18 RNA synthesis at the nonpermissive temperature by PCV305. Blots were probed with mu18. Upper blot represents cells incubated at 33°C; lower blot represents cells incubated at 39°C. (B) PCV305 positive control at 33 and 39°C. Blots were probed with WB1. (C) Probe specificity controls. Probes WB1 and mu18 were tested for ability to hybridize to the mock-infected, PCV305-infected, or 3D-18-infected cells under the hybridization conditions used for the blots shown in panels A and B. PCV305 and 3D-18 were both grown at 33°C.

viruses with similar plaque phenotypes cannot be compared by using this method.

In our approach, cells were infected with either 3D-18, the pseudo-wild-type virus PCV305, or both simultaneously. PCV305 (21) differs from wild-type PV1(M) only in the 5'NCR, where a homologous substitution of the first 627 nt with those of coxsackievirus B3 has been engineered. The growth phenotype of PCV305 is wild type. Infected cells were harvested within 7 to 8 h postinfection, and total cellular RNA was subjected to slot blot hybridization using strand-specific oligonucleotide probes. One probe, L391, hybridized specifically to PV1 5'NCR sequences and thus was able to detect 3D-18 RNA but not PCV305 RNA in cells coinfected with both viruses. Using this probe, we demonstrated rescue of 3D-18 RNA replication by PCV305 at the nonpermissive temperature.

Approximately 6,500 nt separate the L391 complementary region of viral RNA from the site of the 3D-18 lesion at nt 6756. Although we thought it unlikely that our assay would detect rare recombination events occurring between the L391 complementary region in 3D-18 and the wild-type nt-6756 region of the coinfecting PCV305 during the short time course of the experiment, we nevertheless repeated the rescue experiment using probe mu18, complementary to the region around nt 6756 in 3D-18. Probe mu18 unequivocally detected mutant but not PCV305 RNA in the control single infections at the permissive temperature. At the nonpermissive temperature, 3D-18 RNA was detected at significant levels only in the coinfection, confirming that rescue rather than recombination had been observed.

Is there a *cis*-acting protein determinant in poliovirus replication? Bernstein et al. (3) concluded that such a determinant might exist in 2B or 3D, but the failure to detect complementation of 2B or 3D in a plaque assay could be due simply to the low sensitivity of the assay. Moreover, the positive detection of complementation of a small lesion in, for example, 2C does not necessarily mean that all functions of 2C, if it is polyfunctional, may be *trans* complementable. Sarnow et al. (37) did show that the 3'NCR was *cis* acting in replication, but that was not unexpected, since the 3'NCR does not encode a diffusible product. Rather, the 3'NCR is likely to be important in the initiation of minus-strand synthesis.

Hagino-Yamagishi and Nomoto (16) and Kuge et al. (24) have provided evidence that a *cis*-acting replication determinant maps somewhere in the P2 or P3 region. However, their data argue only that disruptions of the viral reading frame upstream of the P1/P2 junction produce nonreplicating, nonrescuable RNA templates. While this is suggestive of *cis* dependency, there are other ways of interpreting their data. It is possible, for example, that ongoing translation of an RNA template opens up an RNA secondary structure or in some way exposes an RNA sequence required for initiation of RNA synthesis. The frame-shifted RNA template would terminate translation just downstream of the frameshift, and the hypothetical secondary structure would remain closed. In that case, the *cis* determinant might well be said to map to a region in P2 or P3, but it would act at the RNA level rather than at the protein level. Indeed, a recent demonstration of the formation in vitro of a covalent linkage between poliovirus RNA and synthetic VPg in the absence of other viral or cellular proteins provides evidence for *cis*-acting replication determinants that act at the RNA level (40).

It should be noted that Agut et al. (1) demonstrated that a guanidine-resistant mutant which was temperature sensitive for growth and RNA synthesis could be rescued in *trans* at the nonpermissive temperature in the presence of guanidine by wild-type (guanidine-sensitive) virus. The temperature-sensitive growth phenotype of the mutant was mapped to a region of 3D containing two nucleotide changes. The plaque assay that the authors used to detect rescue did not rule out the possibility that recombination between wild type and mutant had produced guanidine-resistant $3D^+$ plaques. Also, it is possible that rather than wild type rescuing mutant in their system, the guanidine resistance function (2C?) of the mutant actually *trans* rescued wild-type growth at 39°C in the presence of guanidine, a result which would be interesting in its own right. Because their mutant polymerase activity in vitro was not temperature sensitive even though [^{3}H]uridine incorporation was temperature sensitive in vivo, the authors concluded that the rescuable RNA synthesis defect acted at the level of initiation.

In the context of these earlier results, our data provide strong evidence that at least one activity of 3D is *trans* acting in vivo. However, this conclusion does not rule out the presence of *cis*-dependent functions that map to 3D. In addition to its role in RNA synthesis initiation (10, 41) and elongation (15) and in P1 protein processing (22, 43), 3D could be involved in controlling the ratio of plus to minus strands. It could also play a role in clearing the RNA plus-strand template of ribosomes so that minus-strand synthesis, which utilizes the same template but proceeds in the opposite direction of translation, can take place. Finally,

Andino et al. (2) have demonstrated that 3D in the presence of 3C and host cell proteins binds specifically to conserved secondary structure motifs in the 5'NCR of poliovirus plus strands. The functional significance of this binding is unknown.

In summary, we have identified and characterized a temperature-sensitive elongation mutant, 3D-18, which differs from wild type by the insertion of 3 nt at position 6756 in the 3D coding region. We have also developed a highly sensitive and specific method of analyzing the ability of mutant RNA templates to replicate in the presence or absence of helper functions provided in *trans*. Finally, we have used this method to demonstrate that the defect in 3D-18 is *trans* rescuable. Applied to other mutations, this approach should be useful in defining the *cis*-dependent loci in poliovirus replication if, indeed, they exist.

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