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Mutation of Lysine Residues in the Nucleotide Binding Segments of the Poliovirus RNA-Dependent RNA Polymerase

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The poliovirus 3D RNA-dependent RNA polymerase contains two peptide segments previously shown to cross-link to nucleotide substrates via lysine residues. To determine which lysine residue(s) might be implicated in catalytic function, we engineered mutations to generate proteins with leucine residues substituted individually for each of the lysine residues in the NTP binding regions. These proteins were expressed in *Escherichia coli* and were examined for their abilities to bind nucleotides and to catalyze RNA chain elongation in vitro. Replacement of each lysine residue in the NTP binding segment located in the central portion of the 3D molecule (Lys-276, -278, or -283) with leucine produced no impairment of GTP binding or polymerase activity. Substitution of leucine for Lys-61 in the N-terminal portion of the protein, however, abolished the binding of protein to GTP-agarose and all detectable polymerase activity. A nearby lysine replacement with leucine at position 66 had no effect on enzyme activity. The three mutations in the central region of 3D were introduced into full-length viral cDNAs, and the infectivities of RNA transcripts were examined in transfected HeLa cells. Growth of virus containing 3D with a mutation at residue 278 (3Dμ278) or 3Dμ283 was indistinguishable from that of the wild type; however, 3Dμ276 generated extremely slow-growing, small-plaque virus. Polyprotein processing by 3CDμ276 was unaffected. Large-plaque variants, in which the Leu-276 codon had mutated again to an arginine codon, emerged at high frequency. The results suggest that a lysine residue at position 61 of 3D^{pol} is essential for polymerase catalytic function and that a basic (lysine or arginine) residue at position 276 is required for some other function of 3D important for virus growth but not for RNA chain elongation or polyprotein processing.

The RNA-dependent RNA polymerase (3D^{pol}) of poliovirus is a 52-kDa protein cleaved from the C-terminal end of the poliovirus polyprotein. It catalyzes chain elongation of viral RNA in virus-infected cells. The enzyme has been extensively purified from poliovirus-infected cells (33), and its cDNA has been cloned and expressed in bacterial and insect cells, facilitating the purification of large amounts of enzyme from these sources (18, 20, 21, 25, 27). The polymerase exhibits an RNA chain elongation activity that is dependent upon an RNA template and a DNA or RNA primer (for reviews, see references 16, 23, and 29).

Several amino acid sequences are conserved among all RNA-templated polymerases (22), most likely reflecting their shared catalytic functions and serving as a signature to identify RNA-dependent polymerase function. In poliovirus 3D^{pol}, these conserved residues include a YGDD sequence (residues 326 to 329), D-233, and G-289. Three-dimensional structural analyses of several polynucleotide polymerases reveal strikingly similar core structures, which are designated fingers, palm, and thumb domains (15), referring to the resemblance of the core structure to a right hand. The conserved residues in poliovirus 3D^{pol} appear to reside in the palm domain (10a) and are thought to contribute to the catalytic pocket of the polymerase. D-328 and D-329 are residues which are probably involved in metal ion coordination (13). The roles of the other conserved residues are unknown. These enzymes, however, all share properties of template and nucleotide binding, as well as catalysis of phosphodiester bond formation.

We have previously identified two regions in the poliovirus 3D^{pol} molecule that can be cross-linked to oxidized nucleotide substrates (24). Both sites can accommodate all four ribonucleotides, as indicated by nucleotide binding competition experiments. One region is located in the central portion of the molecule (residues 266 to 286), most likely in the fingers domain (10a); the second nucleotide binding site is located near the N terminus of 3D, between residues 57 and 74. The structure of this portion of the 3D protein appears disordered in the X-ray-diffraction map, and its precise folding has not been determined. Nucleotide binding usually involves specific positively charged residues which bind the polyphosphate moiety of NTP (15). No arginine residues are contained in the two NTP binding segments; therefore, we proposed that one of the three lysine residues in the central region peptide (K-276, K-278, or K-283) and one of the two lysine residues in the N-terminal peptide (K-61 or K-66) bind nucleotides and that at least one of these might participate in the catalytic polymerase function.

In this report, we describe experiments in which the lysine residues in the NTP binding regions were individually changed to leucine residues; the results of these changes were monitored to examine effects on GTP binding and on the 3D^{pol} activity of enzyme expressed in *Escherichia coli*. In addition, mutant 3D genes were incorporated into full-length viral cDNAs, and the infectivities of RNA transcripts in transfected HeLa cells were characterized.

MATERIALS AND METHODS

Generation of mutations in lysine residues. Single amino acid substitutions were introduced into 3D^{pol} by overlap extension PCR as described by Ho et al. (12) and Burns et al. (6) with plasmid pEXC-3D, which contains poliovirus 3CD coding sequences (25). In each set of overlapping primers, the code for lysine was changed to a code for leucine (Fig. 1). Secondary PCR amplification with outside primers representing nucleotides (nt) 5594 to 5614 and the complement to nt

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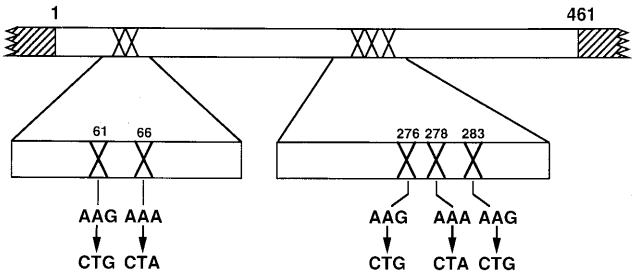

FIG. 1. Lysine-to-leucine mutations in poliovirus $3D^{pol}$. $3D^{pol}$ is depicted as an open bar (1 to 461); expanded regions illustrate peptides containing cross-linked NTP. Lysine residues in the peptide bordered by Ala-57 and Met-74 were mutated to leucine by the indicated nucleotide changes. Similarly, lysine residues in the peptide bordered by Asp-266 and Met-286 were changed to leucine residues.

6299 to 6322 (for mutations at 3D residues 61 and 66) or nt 6416 to 6433 and the complement to nt 7202 to 7220 (for mutations at 3D residues 276, 278, and 283) generated DNA fragments which could be cut with *Bgl*II and *Xba*I for mutations at residues 61 and 66 or with *Nde*I and *Sty*I for mutations at residues 276, 278, and 283. The generated fragments were gel purified and inserted into the vector pEXC-3D (25), from which similarly excised wild-type 3D sequences were removed and vector ends were dephosphorylated with shrimp alkaline phosphatase (U.S. Biochemical). Plasmids were transformed into *E. coli* C600 or JM109; colonies were screened by isolation of plasmid DNA, restriction analysis, and sequencing through mutated regions to confirm retention of mutations.

Expression of 3D proteins in *E. coli*. Single colony isolates of *E. coli* harboring plasmids coding for wild-type or mutant $3D^{pol}$ were expressed at 30°C or, for some experiments, at 37°C in M9 medium (28) until an A_{595} of 1.0 to 1.1 was achieved, and crude sonicates were prepared as described previously (6, 27). Lysates were clarified and fractionated with ammonium sulfate (20) to generate material for assay of polymerase elongation activity (11). Estimation of $3D^{pol}$ levels was accomplished by Western immunoblotting of serial dilutions of bacterial extracts screened with anti-3D serum (26), and 3D activities were compared for similar amounts of 3D protein.

GTP-agarose affinity chromatography of $3D^{pol}$. A 1-ml column of GTP-agarose (Sigma) was equilibrated in buffer A (0.05 M Tris-HCl [pH 8.0], 5 mM β-mercaptoethanol, 0.05 M KCl, 0.1% Nonidet P-40, 10% glycerol) at 4°C. Dialyzed ammonium sulfate fractions (0 to 40% saturation precipitate), or occasionally phosphocellulose (Whatman P11) column fractions, containing $3D^{pol}$ were fractionated on GTP-agarose as described previously (26) with the following modifications. After sample was applied, columns were washed with 6 0.5-ml aliquots of buffer A and bound 3D was eluted in 10 0.5-ml aliquots of buffer A containing 5 mM ATP. Fractionation of polymerase was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Generation of clones containing full-length poliovirus sequences. Plasmid pT7PV1 (10) contains a full-length copy of wild-type poliovirus type 1 downstream of a T7 RNA polymerase promoter. A *Sal*I linker (New England Biolabs) was inserted in pT7PV1 following linearization with *Eco*RI and end filling with the Klenow enzyme. The resulting construct (pT7PV1-*Sal*I) contains a *Sal*I site downstream of the code for the poly(A) tail. The *Bgl*II-*Sal*I segment of pT7PV1-*Sal*I, which contains sequences coding for the C-terminal portion of 3C and all of 3D, was excised and exchanged with the corresponding fragments from pEXC-3D, containing each of the mutations in 3D at residues 276, 278, and 283 (3Dμ276, 3Dμ278, and 3Dμ283, respectively), described above. After ligation, the DNA was transformed into *E. coli* C600. Plasmids from isolated colonies were verified by restriction mapping and sequencing in the region of each mutation to confirm retention of the correct constructs.

Transcription, RNA transfection, and virus isolation. pT7PV1-*Sal*I, which contains wild-type 3D sequences or mutations at amino acid 276, 278, or 283 in 3D, was linearized with *Sal*I, and these DNAs were transcribed by T7 RNA polymerase in in vitro transcription reactions with a T7 MEGAscript kit from Ambion, Inc., according to the protocol of the manufacturer. RNA concentrations were estimated by agarose gel analysis and ethidium bromide staining of the transcripts. HeLa cell monolayers were grown to confluency in minimal essential medium supplemented with 8% fetal calf serum, 1% glutamine, 1% antibiotic-antimycotic (penicillin G, streptomycin, amphotericin B; Gibco BRL), and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4), in six-well dishes (Falcon; 30 mm per well). Transcripts were transfected by the DEAE-dextran (Pharmacia) method (31, 32), cells were covered with a semisolid agar layer (0.44% agarose [FMC SeaPlaque], minimal essential medium, 6% fetal calf serum, 1% antibiotic-antimycotic), and incubation was maintained at 37°C.

Two days (wild type, 3Dμ278, or 3Dμ283) or 3 days (3Dμ276) posttransfection, virus plaques were picked and dispersed in 200 μl of minimal essential medium, frozen and thawed three times, and centrifuged at 900 × *g* for 5 min. The supernatant served as a virus stock. Stocks were either amplified by further passage in HeLa cell monolayers or used to infect cells for RNA isolation and sequencing.

In vitro translation of viral RNAs. Transcripts of viral cDNAs were purified, after digestion of residual DNA with DNase I, by phenol-chloroform extraction and ethanol precipitation. These transcripts were translated in HeLa S10 extracts prepared as described by Molla et al. (17) and Barton et al. (3) at 30°C for 6 h to allow for polyprotein processing.

RESULTS

Construction of mutations in $3D^{pol}$. In a previous study, we identified two internal peptides in poliovirus $3D^{pol}$ that each contained lysine residues which specifically cross-linked to oxidized NTP (24). One site was located between Ala-57 and Met-74, suggesting that Lys-61 or Lys-66 represented one NTP binding site (Fig. 1). A second site was located between Asp-266 and Met-286, with Lys-276, Lys-278, or Lys-283 as an implicated site. In order to determine which, if any, of these lysine residues contributed to the catalytic function of the enzyme, we engineered site-specific mutations to generate proteins with a leucine residue substituted individually for each of the implicated lysine residues. These proteins could then be examined for their abilities to bind nucleotides and for their catalytic activities in RNA chain elongation. In addition, the effect of each individual mutation on the infectivity of full-length viral RNA could be tested. Two nucleotide changes were introduced to replace specific lysine residues with leucine (AAA or AAG→CTA or CTG). These mutations were cloned into the expression plasmid pEXC-3D by exchange of wild-type sequences with PCR-generated segments. Immunoblot analysis of the proteins produced in *E. coli* transformed with the wild type and each mutant plasmid demonstrated the expected expression of 3CD, with approximately 70% cleavage to generate 3D (Fig. 2). Additional immunoreactive bands, likely representing degradation products or premature termination products, were detected at relatively low levels. Some variability was seen in the patterns and relative amounts of these bands, such as is shown for μ61 in Fig. 2, but the variation was not reproducibly specific for any particular mutation. None of the mutations introduced into the 3D sequence appeared to affect the cleavage of 3CD.

Affinity of $3D^{pol}$ for GTP-agarose. Affinity column chromatography on GTP-agarose is a convenient measure of NTP

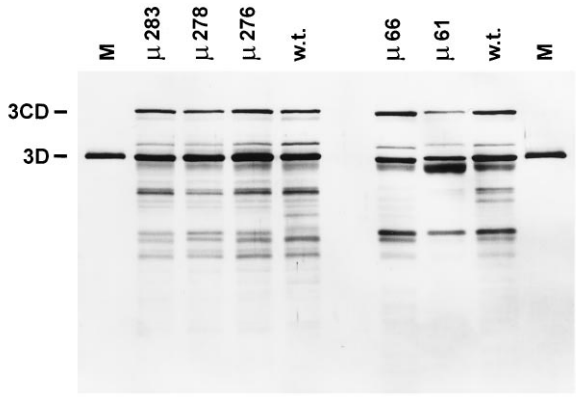

FIG. 2. Immunoblot of ammonium sulfate-fractionated extracts containing wild-type and mutant $3D^{pol}$. Expression of $3D^{pol}$ containing wild-type and mutated sequences from plasmid pEXC-3D was examined following ammonium sulfate fractionation of clarified bacterial cell extracts. Blots were probed with anti-3D serum after SDS–10% PAGE fractionation of proteins. Lanes labeled M contain purified $3D^{pol}$ marker. Other lanes contain preparations of the indicated wild-type (w.t.) and mutant $3D^{pol}$.

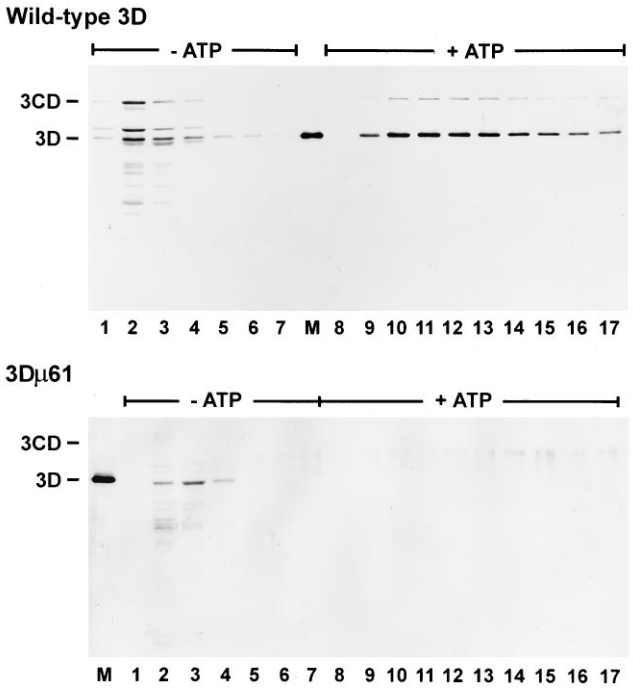

FIG. 3. Immunoblots of GTP-agarose column fractions of wild-type and 3Dμ61 preparations. Ammonium sulfate-fractionated preparations of wild-type $3D^{pol}$ (top panel) and 3Dμ61 (bottom panel) were applied to GTP-agarose columns, and flowthrough (− ATP) and eluted (+ ATP) fractions were obtained, fractionated in SDS–10% polyacrylamide gels, and analyzed by immunoblotting with anti-3D serum. Numbered lanes in each immunoblot indicate successive fractions from the GTP-agarose column; M denotes purified marker $3D^{pol}$.

binding by $3D^{pol}$. Wild-type $3D^{pol}$ has been shown to bind to GTP-agarose (26). When preparations of wild-type 3D were applied to GTP-agarose columns, essentially all of the polymerase activity and the majority of anti-3D immunoreactive protein remained bound to the column, whereas a minor but variable fraction of immunoreactive protein flowed through the column matrix (26) (Fig. 3, top panel). Bound material was eluted with either ATP or GTP. 3D protein that failed to bind to GTP-agarose was interpreted to represent a portion of denatured enzyme in the preparation, since it displayed no detectable polymerase activity (data not shown). To examine the ability of mutated $3D^{pol}$ containing leucine residues at positions 61, 66, 276, 278, and 283 to bind GTP, ammonium sulfate-fractionated *E. coli* extracts were subjected to GTP-agarose affinity chromatography, and the flowthrough and eluted fractions were analyzed by anti-3D immunoblotting. All of the mutant proteins showed the same binding and elution pattern as the wild-type enzyme (data not shown), except protein with the Lys-61→Leu replacement, which failed to bind to the column at all (Fig. 3, bottom panel). Slightly less mutant protein was applied to the column shown in Fig. 3, so the uncleaved 3CD protein was not detected in this experiment. These results suggested that the GTP binding site in 3Dμ61 was not present or not available to the resin, whereas all other lysine-to-leucine replacements did not affect the ability of the proteins to bind GTP.

Elongation activity of $3D^{pol}$. $3D^{pol}$ was expressed from the wild type or mutant pEXC-3D plasmids in *E. coli* as described previously (27). Clarified *E. coli* extracts were fractionated by 40% ammonium sulfate saturation, and RNA polymerase elongation activities were determined in a poly(U) polymerase assay with a poly(A) template. Assays were conducted at 30°C, since the level of $3D^{pol}$ activity is reduced three- to fivefold in 30 min at 37°C compared with that at 30°C (unpublished results). The assays were performed on equal amounts of $3D^{pol}$ protein in each sample as estimated by fractionation of serial dilutions of the ammonium sulfate fractions by SDS-PAGE and then by immunoblotting with anti-3D serum. The results of the assays for the wild type and site-specific mutants at positions 276, 278, and 283 in the 3D molecule are shown in Fig. 4A. All mutant enzymes displayed wild-type levels of activity at 30°C, demonstrating that none of these three lysine residues was specifically required for polymerase elongation activity. Similar analyses were performed with proteins containing leucine at positions 61 and 66, and the results of these assays

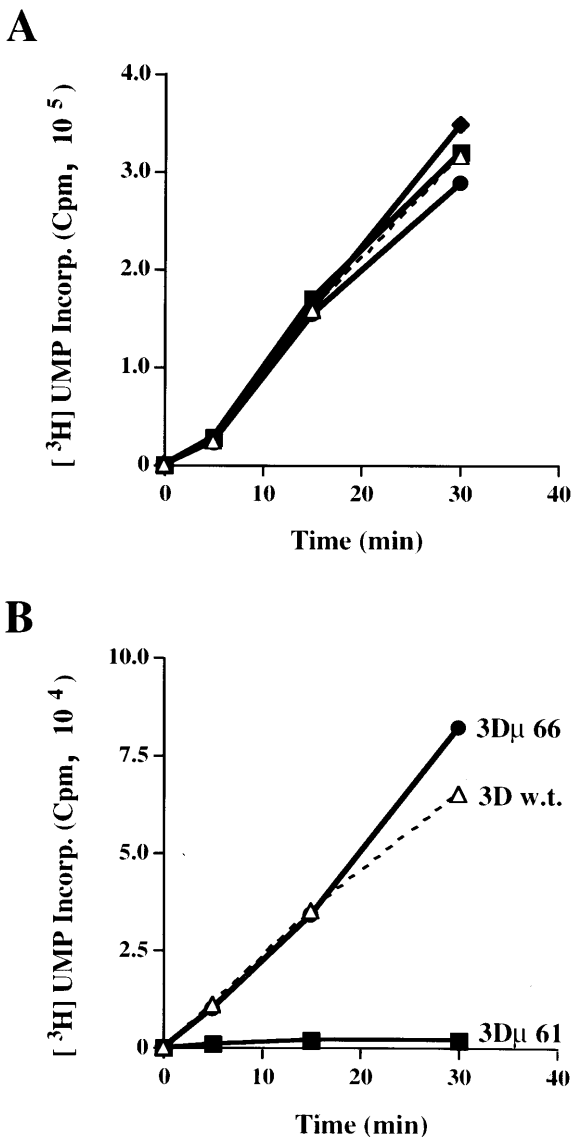

FIG. 4. Elongation activities of wild-type 3D and mutant 3D proteins. Poly(U) polymerase activities in ammonium sulfate-fractionated extracts containing wild-type and mutant $3D^{pol}$ were measured. Activities are plotted for preparations containing similar amounts of $3D^{pol}$, as determined by immunoblot analysis. (A) Wild-type 3D and 3Dμ276, 3Dμ278, and 3Dμ283 activities were measured as a function of time. --△--, wild type; —■—, 3Dμ276; —◆—, 3Dμ278; —●—, 3Dμ283. (B) Wild-type (w.t.) 3D, 3Dμ61, and 3Dμ66 activities are shown. Incorp., incorporated.

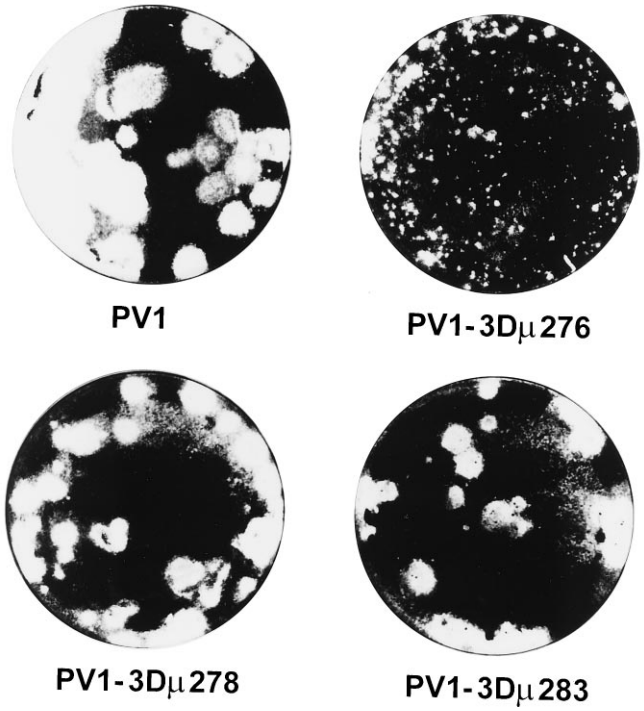

FIG. 5. Plaque morphologies of wild-type poliovirus (PV1) and viruses containing 3Dμ276, 3Dμ278, or 3Dμ283. Plaques were formed on HeLa cell monolayers after transfection with full-length transcripts of poliovirus cDNAs. Plates were incubated at 37°C for 2 days and stained with crystal violet.

are shown in Fig. 4B. The lysine-to-leucine mutation at residue 66 did not alter 3D polymerase activity; mutation at position 61, however, resulted in the loss of all detectable RNA chain elongation activity (less than 5% of the level of wild-type activity). These results are consistent with the GTP binding data described above; 3Dμ61 showed no GTP binding and no polymerase activity; none of the other mutations affected GTP binding or polymerase activity.

Transfection of HeLa cells with full-length transcripts containing 3Dμ276, 3Dμ278, and 3Dμ283. Mutated 3D sequences from pEXC-3Dμ276, -3Dμ278, and -3Dμ283 were substituted for the corresponding sequence in pT7PV1, a full-length infectious cDNA clone driven by a T7 promoter (10). Confluent monolayers of HeLa cells were transfected with RNA transcripts of the wild type and each mutant cDNA and overlaid with agar, and plaques were allowed to develop over 2 to 3 days. Figure 5 shows the morphologies of the plaques resulting from each transcript. The plaques induced by 3Dμ278 and 3Dμ283 appeared identical to those of the wild type, reaching about 4 to 5 mm in two days. 3Dμ276 produced minute plaques at 2 days, which continued to grow slowly during further incubation. Six isolated plaques developed for 3 days from transfections with transcripts of two independent 3Dμ276 clones were used to infect fresh monolayers of HeLa cells, and total cellular RNA was isolated after 6 h of infection, amplified by reverse transcription-PCR, and sequenced. The mutations generating 3Dμ276 were recovered in all of the small-plaque viruses. Occasionally, a large plaque appeared on these plates, suggesting that reversion to the wild-type phenotype occurred.

Accordingly, HeLa cell monolayers were transfected with full-length 3Dμ276 RNA transcripts under a liquid medium overlay, and an amplified virus stock was generated after lysis of the monolayer began. Cytopathic effects were observed after about 60 to 70 h, compared with 24 h for wild-type virus. The amplified virus stock was then used in a plaque assay on fresh monolayers of HeLa cells. After 2 days, numerous large plaques that were indistinguishable from plaques of wild-type virus had developed. This rapid emergence of large-plaque variants from 3Dμ276 virus implied that mutations were generated during the first and second virus passages. Five large plaques were picked for further characterization. The 3D coding region from nt 6416 to 7220 was amplified by reverse transcription-PCR, and the resulting DNA fragments were subjected to dideoxy sequencing. Table 1 shows the sequences recovered. In all five revertant clones, the CTG leucine codon that had been inserted to replace the AAG lysine codon in the wild-type sequence was replaced by a CGG codon, specifying arginine. Apparently, a lysine-to-arginine change in position 276 of the 3D sequence generated viral proteins able to carry out all functions of the wild-type proteins at normal or near-normal rates. In some plaques, both mutant and revertant viruses were recovered (R3 and R5), indicating that the reversion event may have occurred during the growth of virus in the plaque or possibly that the large plaque was contaminated with one or more small plaques, which were sometimes difficult to see.

Further characterization of 3Dμ276. The defective growth phenotype of viruses containing 3Dμ276 was unexpected, since the RNA polymerase activity of 3Dμ276 protein produced in *E. coli* was indistinguishable from that of the wild type. Since plaques from the mutant virus were developed at 37°C while bacteria expressing 3D protein were generally grown at 30°C, we were concerned that the mutation at position 276 might generate an enzyme that was susceptible to folding errors when synthesized at the elevated temperature. To examine the activity of 3Dμ276 synthesized at 37°C, bacteria carrying pEXC-3D or pEXC-3Dμ276 were induced for expression of 3D sequences at 37°C and extracts were prepared for assay. No reduction in the level of mutant 3D activity compared with that of the wild type was observed (data not shown).

Complete processing of the viral polyprotein precursor requires proteolytic cleavage catalyzed by 3CD protease. Although the nonstructural viral proteins can be generated by 3C-mediated cleavages alone, processing of the capsid proteins requires the presence of 3D sequences in the 3CD protease for efficient substrate recognition (14, 34). An alternative explanation for the poor growth displayed by virus containing 3Dμ276 was that the mutant 3CD was defective for capsid protein processing rather than for RNA synthesis. To examine the protein processing activity of 3CDμ276, RNA transcripts of wild-type and 3CDμ276 sequences in pT7PV1 were translated in HeLa cell extracts optimized for translation of poliovirus RNA (3, 17). Figure 6 shows that both wild-type and 3CDμ276 RNAs were translated efficiently and generated the same spectrum of viral proteins, including capsid proteins VP0, VP1, and VP3 (compare lane 2 with lanes 3 and 4). Thus, protein pro-

TABLE 1. Sequences recovered in large-plaque revertants of virus containing 3Dμ276

Virus isolate	3D276 codon	Amino acid
Wild type	AAG	Lys
μ276	CTG	Leu
R1	CGG	Arg
R2	CGG	Arg
R3	CT, GG	Leu, Arg
R4	CGG	Arg
R5	CT, GG	Leu, Arg

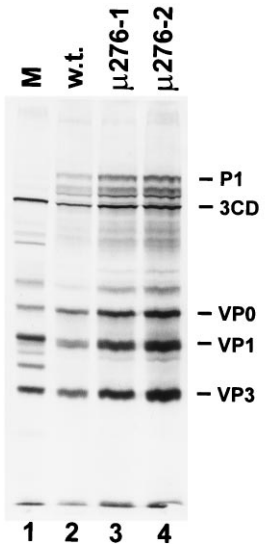

FIG. 6. Translation of wild-type (w.t.) and 3Dμ276 RNAs in HeLa cell S10 extracts. Extracts containing ^{35}S-labeled proteins synthesized in HeLa cell extracts were fractionated in an SDS–10% polyacrylamide gel and analyzed by autoradiography. Lane 1 (M) contains an infected HeLa cell extract labeled with [^{35}S]methionine from 2 to 4 h postinfection. Lanes 2, 3, and 4 contain extracts obtained after translation of transcripts from wild-type, 3Dμ276 (clone 1), and 3Dμ276 (clone 2) cDNAs, respectively. Specific viral proteins are indicated to the right.

cessing defects are not responsible for the poor growth of 3CDμ276.

DISCUSSION

Binding of nucleotide substrates to polynucleotide polymerases is a prerequisite for catalysis of chain elongation. Interaction of the protein with a nucleotide substrate usually involves a positively charged residue and the negatively charged polyphosphate moiety of the nucleotide (15). In a previous study, we utilized a lysine-dependent cross-linking procedure to identify nucleotide binding sites in the poliovirus RNA polymerase. We isolated two peptide sequences that had been cross-linked to nucleotides. One of these sequences spanned residues 57 to 74, which contained Lys-61 and Lys-66; the second spanned residues 266 to 286, which contained Lys-276, Lys-278, and Lys-283. The latter peptide is located within a conserved motif (motif B) (22) that contains a glycine residue common to all RNA-dependent RNA polymerases. The former peptide, close to the N terminus of the protein, lies outside the core region which manifests sequence conservation among RNA-templated polymerases, but its location within the three-dimensional structure of the enzyme has not been determined. Binding of nucleotides to multiple sites in polymerases, in which only one site is the functional binding site in the catalytic process, is not uncommon. For example, Basu and Modak (5) found that dTTP protected only one (Lys-758) of four pyridoxal phosphate binding sites in Klenow polymerase. Also, in murine leukemia virus, reverse transcriptase dNTP binding protected two sites from pyridoxal phosphate binding, but mutation of these sites showed that only one site (Lys-103) had a significant effect on enzyme activity (4).

To distinguish which of the multiple lysine residues in each of the two peptides that cross-link to nucleotides were involved in catalytic activity, we applied a molecular genetic strategy in which each lysine was individually changed to a leucine residue and the effects of each mutation on the NTP binding properties, as well as the polymerase activity of each protein, were evaluated. Replacement of each lysine residue individually in the peptide spanning residues 266 to 286 had no effect on GTP binding or polymerase activity, suggesting that NTP binding to Lys-276, -278, or -283 is not required for these functions of the protein. It is possible that binding to a lysine residue in the peptide is essential, but elimination of one lysine induces or allows interaction of a nucleotide with another nearby lysine. We did not attempt to construct multilysine mutations in this peptide, however, because removal of two or three positively charged amino acids from a small region of the protein would likely have a significant effect on the overall folding of the molecule, which would complicate the interpretation of any observed loss in protein function.

Replacement of Lys-61 with leucine caused a complete loss of the protein's affinity for GTP-agarose and of its polymerase activity. These data suggest a functional involvement of this lysine in catalytic activity. Although an alteration of the overall conformation of the protein, resulting in loss of activity, cannot be ruled out, the similar lysine-to-leucine change at nearby position 66 had no discernible effect on activity.

Further support for a functional role of Lys-61 in polymerase function emerges from analysis of alignments of 3D protein sequences from 40 different strains, all of known genera of picornaviruses (20a). Of the 5 lysine residues evaluated in this study (residues 61, 66, 276, 278, and 283), only Lys-61 is absolutely conserved among all 3D proteins. A selected subset of these sequences is shown in Fig. 7. Residues 66, 276, 278, and 283 are highly variable.

Although a number of laboratories have isolated or constructed viruses with mutations in 3D, relatively few have examined mutations in the N-terminal portion of the protein. Plotch et al. (21) produced a deletion of Trp-5 which lost all polymerase activity. A series of mutations of clustered charged amino acids to alanines within the first 100 residues of 3D^{pol} rendered viruses temperature sensitive, with variable losses of RNA accumulation at the nonpermissive temperature (8). For example, 3D containing Lys-51→Ala plus Asp-53→Ala showed a 40% loss of viral RNA accumulation at 39°C whereas 3D containing Asp-53→Ala plus Glu-55→Ala plus Glu-56→Ala showed a 90% loss of viral RNA accumulation. In these cases, however, the activities of the polymerase protein per se were not measured, so it is difficult to correlate the structural changes with function.

3D protein, like most of the picornaviral proteins, likely has multiple functions. Its catalytic role in RNA chain elongation has been well characterized (9, 33). 3D sequences contribute to the function of 3CD, both as a protease for cleavage of capsid protein precursors (14, 34) and as an essential participant in 5′-terminal cloverleaf binding for initiation of viral RNA synthesis (1, 2). It has been suggested that 3D may play a role in VPg uridylylation (30), and several other enzymatic activities for 3D have been observed (7, 19), although their precise functions in RNA replication are uncertain. The availability of 3D mutations with normal chain elongation activities provides an opportunity to screen for defects in other 3D functions. For this reason the three lysine-to-leucine mutations from the central core domain (3Dμ276, 3Dμ278, and 3Dμ283), with normal polymerase activities in vitro, were introduced into expression plasmids containing full-length cDNA. All three mutated sequences generated infectious RNAs that produced viable viruses. Poliovirus 3Dμ276, however, grew extremely slowly, producing only pinpoint-sized plaques 2 days after transfection of HeLa cells. The other two mutants produced plaques indistinguishable from those of wild-type virus. The slow growth of

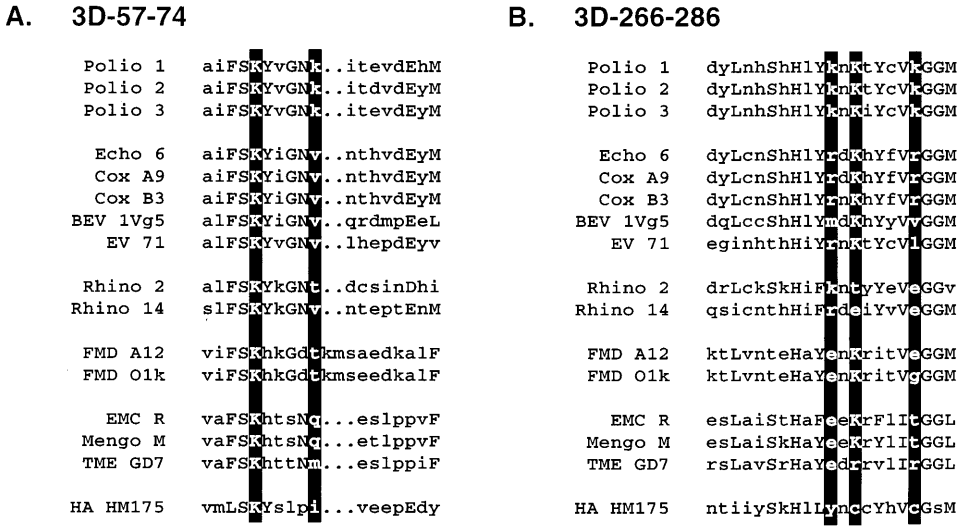

FIG. 7. Sequence comparison of picornaviral 3D peptides. The segments of poliovirus 3D which cross-link to NTP (residues 57 to 74 [A] or 266 to 286 [B]) are compared with similar segments in other picornaviruses representative of the different genera. Residues 61, 66, 276, 278, and 283 in poliovirus 3D are aligned with similar residues in these viruses and are indicated in black boxes. Capital letters denote residues which show a predominant match throughout aligned picornaviral sequences. Polio, poliovirus; Echo, echovirus; Cox, coxsackievirus; BEV, bovine enterovirus; EV, enterovirus; Rhino, rhinovirus; FMD, foot-and-mouth disease virus; EMC, encephalomyocarditis virus; Mengo, mengovirus; TME, Theiler's murine encephalomyelitis virus; HA, hepatitis A virus.

3Dμ276 was not due to defective protein processing by the mutant 3CD. It is possible that 3Dμ276 is defective in a 3D function required for an initiation step of RNA synthesis, which results in its extremely poor growth. Alternatively, the mutated RNA might be a poor substrate for replication, rather than it encoding a defective protein. Unfortunately, analyses of other aspects of RNA synthesis, such as VPg uridylylation, could not be examined in cells infected with 3Dμ276 because of the rapid emergence of revertant viruses during growth. Of the five phenotypic revertants that we isolated following one passage in HeLa cells, all had replaced the leucine codon (CTG) with an arginine codon (CGG), which required only a single nucleotide change. Reversion to the wild-type lysine codon (AAG) would have required two nucleotide changes and was not observed. Apparently, replacement of another positively charged residue at that site was sufficient to confer a wild-type level of activity to the protein.

The results of this study suggest that a lysine residue at position 61 is essential for binding of a nucleotide substrate and catalysis of RNA chain elongation. A basic residue (lysine or arginine) at position 276 appears to be required for a different function of 3D and essential for efficient virus growth but not for RNA chain elongation activity or polyprotein processing.

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