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Poliovirus RNA Polymerase Mutation 3D-M394T Results in a Temperature-Sensitive

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Mutant ts10 is an RNA-negative temperature-sensitive mutant of Mahoney type 1 poliovirus. Mutant ts10 $3D^{pol}$ was purified from infected cells and was shown to be rapidly heat-inactivated at 45° when compared to wild-type polymerase. Sequencing of mutant ts10 genomic RNA revealed a U to C transition at nt 7167 resulting in an amino acid change of methionine 394 of $3D^{pol}$ to threonine. The 3D-M394T mutation was engineered into a wild-type infectious clone of poliovirus type 1. The resultant mutant virus, 3D-105, had a temperature-sensitive phenotype in plaque assays. The translation and replication of wild-type, ts10, and 3D-105 virion RNAs were all characterized in HeLa S10 translation-RNA replication reactions *in vitro.* The optimum temperatures for the replication of the wild-type and mutant viral RNAs in the HeLa S10 translation-replication reactions were 37 and 34°, respectively. To characterize the temperature-sensitive defect in the replication of the mutant RNA, we used preinitiation RNA replication complexes which were formed in HeLa S10 *in vitro* reactions containing guanidine HCl. Negative-strand RNA synthesis in 3D-M394T mutant preinitiation replication complexes was normal at 34° but was rapidly and irreversibly inhibited at 39.5°. To differentiate between the initiation and elongation steps in RNA replication, we compared the elongation rates in mutant and wild-type replication complexes at 39.5°. The results showed that the elongation rates for nascent negative strands in both the mutant and wild-type replication complexes were identical. Therefore, the results indicate that the heat-sensitive step in negative-strand synthesis exhibited by the 3D-M394T replication complexes is in the initiation of RNA synthesis and not in the elongation of nascent chains.

INTRODUCTION

Poliovirus is the prototypic member of the Picornaviridae family of positive-strand RNA viruses. Viruses within this family possess unique and interesting strategies for the expression of their genetic material and the replication of their genomes. The poliovirus genome contains one long open reading frame (Racaniello and Baltimore, 1981; Kitamura *et al.,* 1981) from which the viral proteins are expressed via a polyprotein which is cleaved both co- and posttranslationally by three viral proteases: $2A^{Pro}$, $3C^{Pro}$, and $3CD^{Pro}$ (Harris *et al.,* 1990). The genomic RNA contains a virus-encoded protein, VPg, covalently linked to its 5′ end via a phosphodiester linkage to the tyrosine of VPg (Flanegan *et al.,* 1977; Nomoto *et al.,* 1977; Lee *et al.,* 1977). The precise mechanism by which VPg becomes covalently linked to the viral RNA is not clear but certainly plays an intimate role in the replication of the viral RNA.

The virus-encoded RNA-dependent RNA polymerase, $3D^{Pol}$, was purified from poliovirus-infected cells (Van Dyke and Flanegan, 1980; Young *et al.,* 1987; Flanegan and Baltimore, 1979) and shown to possess template and primer-dependent RNA polymerase activity (Van Dyke *et al.,* 1982; Flanegan and Van Dyke, 1979). In contrast, no polymerase activity is associated with viral protein 3CD, the predominant precursor protein of $3C^{pro}$ and $3D^{Pol}$ (Harris *et al.,* 1992; Van Dyke and Flanegan, 1980). $3D^{Pol}$ has been expressed in bacterial and insect expression systems and found to be functionally indistinguishable from the protein expressed in infected cells (Rothstein *et al.,* 1988; Richards *et al.,* 1987; Neufeld *et al.,* 1991a, 1991b; Plotch *et al.,* 1989). $3D^{Pol}$ is also functionally expressed by translation *in vitro* (Barton and Flanegan, 1993). The reconstitution of polymerase activity *in vitro* is not specific for viral RNA, as most template:primer pairs are competent for elongation *in vitro* (Tuschall *et al.,* 1982). The authentic initiation of viral RNA synthesis requires numerous viral proteins, as well as unidentified host proteins (Barton *et al.,* 1995), and has not been achieved in reconstitution reactions containing purified proteins and viral RNA. Authentic viral RNA replication does occur *in vitro* in HeLa S10 translation–replication reactions (Barton *et al.,* 1995; Molla *et al.,* 1991) and these reactions may facilitate the characterization of viral

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and cellular proteins involved in the replication of poliovirus RNA (Shiroki *et al.,* 1993; Kaplan *et al.,* 1989; Barton *et al.,* 1995).

A number of genetic approaches have been used to study the role of $3D^{Pol}$ in viral RNA replication. Toyoda *et al.* (1987) demonstrated that temperature-sensitive mutations within the $3D^{Pol}$ gene of the Sabin strain of type 1 virus affected VPgpUpU formation, although it remains to be determined whether $3D^{Pol}$ catalyzes the uridylylation of VPg (or 3AB) directly. Single amino acid insertion mutations within the $3D^{Pol}$ gene result in the expression of enzymatically inactive $3D^{Pol}$ and defective 3CD protease (Burns *et al.,* 1989), as the $3D^{Pol}$ gene encodes functions other than polymerase activity in the 3CD protein. In one case, an insertion mutation in the $3D^{Pol}$ gene generated a small plaque mutant which was found to be cis-acting in complementation studies and suggested that the polymerase may have a cis-active function during replication (Bernstein *et al.,* 1986). In contrast, two separate studies examined temperature-sensitive mutations within the $3D^{Pol}$ gene which were complemented *in trans* (Charini *et al.,* 1991; Agut *et al.,* 1989). In addition, Novak and Kirkegaard demonstrated the ability to complement an amber mutation which blocked the translation of most of the $3D^{Pol}$ gene (Novak and Kirkegaard, 1994). Using clustered charged-to-alanine mutagenesis, Diamond and Kirkegaard generated numerous mutations within the $3D^{Pol}$ gene in an attempt to generate additional temperature-sensitive mutants (Diamond and Kirkegaard, 1994). They found that all but one of the temperature-sensitive mutants generated in their study mapped in a 75 amino acid long domain between amino acids 50 and 150 (Fig. 10). The other temperature-sensitive mutation generated in their study mapped within one amino acid of the mutation in ts 035 identified by Agut *et al.* (1989). A number of clustered charged-to-alanine mutants were unable to produce virus, indicating lethal mutations (Diamond and Kirkegaard, 1994). The core polymerase domain of $3D^{Pol}$ was identified in studies examining conserved sequences among RNA polymerases (Poch *et al.,* 1989) and comparison of these conserved motifs with the known structure of Klenow DNA-dependent DNA polymerase (Delarue *et al.,* 1990). Jablonski and Morrow (1993) found that mutagenesis of the highly conserved YGDD motif within the evolutionarily conserved core polymerase domain were predominantly lethal; however, an FGDD mutant was viable and a mutation to MGDD was viable in the context of a second site mutation at amino acid 105 of the $3D^{Pol}$.

Here, we describe a M394T mutation within the $3D^{Pol}$ gene of poliovirus mutant ts10 that mapped within a 39 amino acid long region of $3D^{Pol}$ that contained only lethal clustered charged-to-alanine mutants carboxy-terminal to the core polymerase domain (Fig. 10). Mutant ts10 was originally generated by hydroxylamine mutagenesis and characterized as an RNA-negative mutant (Hewlett *et al.,* 1982). We show that ts10 $3D^{Pol}$ was rapidly heat-inactivated as compared to wild-type polymerase. After engineering the 3D-M394T mutation into a wild-type cDNA clone, we isolated mutant virus 3D-105 and found it to be phenotypically indistinguishable from ts10 virus. In HeLa S10 RNA replication reactions, both 3D-105 and ts10 mutant RNAs had a reduced optimum temperature for RNA replication and were strongly inhibited in their ability to initiate RNA replication at the restrictive temperature.

MATERIALS AND METHODS

Virus and cell cultures

Suspension cultures of HeLa S3 cells in Joklik modified Eagle medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% calf serum and 2% fetal calf serum were maintained at 2×10^5 to 4×10^5 cells per milliliter. Cells were infected with poliovirus type 1 (Mahoney), ts10, or 3D-105 viruses as previously described (Villa-Komaroff *et al.,* 1974). Mutant ts10 virus was obtained from Dr. Martinez J. Hewlett at the University of Arizona who originally isolated the ts10 mutant from a stock of poliovirus type 1 (Mahoney) following chemical mutagenesis with hydroxylamine (Kuchta *et al.,* 1988). Plaque assays were performed using monolayers of BSC 40 cells grown in Dulbecco's modified minimum essential media (DMEM) supplemented with 5% calf serum and 2% fetal calf serum. BSC 40 cell monolayers were used for all plaque assays in this study, since we consistently observe larger plaques than with HeLa cell monolayers incubated for the same time. Virus stocks were diluted in PBS (135 m*M* NaCl, 2.5 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, (pH 7.4)) and were allowed to adsorb to PBS-washed BSC 40 cell monolayers for 30 min at 33 or 39°. The monolayers were overlayed with media containing 1% methylcellulose and incubated at 33° for 3 days or 39° for 2 days. Plaques were visualized by fixing and staining the monolayers with 0.02% crystal violet, 2% ethanol, 0.008% ammonium oxalate.

Polymerase purification

The poliovirus RNA polymerase was purified from HeLa cells at 5 hr postinfection as described (Young *et al.,* 1986). Briefly, a high speed supernatant (200,000 *g*) was prepared from a cytoplasmic extract of the infected cells (Fraction I), and the polymerase was precipitated with 35% ammonium sulfate (Fraction II). After chromatography on phosphocellulose, the peak fractions containing the polymerase activity were concentrated against solid sucrose and stored at −70° (Fraction III, 0.5 μg/μl). Mutant ts10 RNA polymerase was purified from HeLa cells infected at 33° for 8 hr using the same protocol. At each stage of purification, polymerase activ-

ity was assayed using a poly(A) template and an oligo(U) primer as described (Tuschall *et al.,* 1982).

Heat-inactivation of polymerase

Aliquots (5 μl) of purified polymerase were heated at 45° for the indicated time, centrifuged for 5 sec in a microcentrifuge and then placed in ice. A standard reaction mixture containing either poly(A):oligo(U) (Tuschall *et al.,* 1982) or poliovirion RNA:oligo(U) (Young *et al.,* 1986) was added to each tube of heated polymerase and polymerase activity was assayed at 25° for 1 hr. Polymerase activity was assayed at 25° in these experiments since this was the optimum temperature for the mutant ts10 polymerase. This was in contrast to the 30° optimum for wild-type polymerase (Flanegan and Van Dyke, 1979). The total amount of [^{3}H]UMP incorporated into product RNA was determined by collecting the labeled product on membrane filters after precipitation with 10% trichloroacetic acid and 2% sodium pyrophosphate. The filters were counted in 5 ml Ready-Solv (Beckman Instruments) scintillation fluid using a Beckman LS5801 liquid scintillation counter.

RNA sequencing

The 3D^{Pol} coding sequence of mutant ts10 virion RNA was sequenced using the dideoxynucleotide primer-extension method (Sanger *et al.,* 1977) with modifications. The virion RNA was isolated as described (Young *et al.,* 1986). Oligonucleotide primers were synthesized using an Applied Biosystems 380A DNA synthesizer and processed according to the protocol of the manufacturer. All synthetic primers were 21 nucleotides long and were constructed at 250 base intervals across the consensus polymerase coding sequence (Kitamura *et al.,* 1981; Racaniello and Baltimore, 1981). Purified poliovirion RNA (1 μg) from either mutant ts10 or Mahoney strain virus was incubated with 5 ng of synthetic primer (1:2 molar ratio) at 60° for 10 min in 10 μl of 34 m*M* Tris:HCl (pH 8.3), 50 m*M* NaCl, 6 m*M* $MgCl_2$, 5 m*M* DTT and then at 42° for 30 min. The annealed template:primer was then reverse transcribed using 1.25 U of AMV reverse transcriptase (Promega) and the appropriate dideoxynucleoside and ^{35}S-labeled deoxynucleoside triphosphates at 42° for 15 min. The labeled transcripts were electrophoresed through a 6% polyacrylamide–7 *M* urea sequencing gel. The gel was dried and exposed to Kodak XAR5 film for 72 hr at −70°.

Construction of 3D^{Pol}-M394T mutant virus

The 3D^{Pol}-M394T mutation was engineered into an infectious poliovirus cDNA clone, pMluN, using oligonucleotide-directed mutagenesis as described (Diamond and Kirkegaard, 1994). The mutation identified in ts10 RNA was a U to C transition at nucleotide 7167 which resulted in an AUG codon (M394 in 3D^{Pol}) being changed to an ACG codon (T394) (Fig. 3). The mutant oligonucleotide (5′GAATTTCCTTAGTTGGCATTAC3′) used to construct the 3D^{Pol}-M394T mutation changed both nucleotides 7167 (U to C) and 7168 (G to U) of the wild-type poliovirus clone, resulting in the alteration of the AUG codon (methionine) to an ACU codon (threonine) (Fig. 3). The two-base change was engineered to yield mutant virus with a lower reversion frequency. A subclone of the 3D^{Pol}-encoding sequence was created by digesting pMlu DNA with *Pvu*II and *Eco*RI and subcloning the resulting segment (nucleotides 7053–7512) into pBluescriptKS + DNA (Stratagene). Deoxyoligonucleotide mutagenesis was performed using the method of Kunkel (1985) and modified as described (Diamond and Kirkegaard, 1994). The 3D^{Pol} sequence was removed from the mutagenized plasmid by digestion with *Pvu*II and *Eco*RI and was cloned into the infectious cDNA clone pMluN. The mutant plasmid was designated as 3D-105. Infectious virus was obtained after transfection of plasmid 3D-105 DNA into HeLa cells at 33° as previously described (Diamond and Kirkegaard, 1994). Two separate isolates (1-B and 12-J) were amplified to create virus stocks and checked for their ability to replicate at both the permissive (33°) and nonpermissive (39°) temperatures. The presence of the appropriate mutations at nucleotides 7167 and 7168 in 3D-105 virion RNA was verified by sequencing.

HeLa S10 translation–RNA replication reactions

Ts10, 3D-105, and Mahoney virion RNAs were added to HeLa S10 translation–RNA replication reactions as described (Barton *et al.,* 1995). The standard 50-μl HeLa S10 translation–RNA replication reaction contained 25 μl HeLa S10 extract, 10 μl translation initiation factors from a salt wash of HeLa cell ribosomes, 5 μl of 10× reaction mix (10 m*M* ATP, 2.5 m*M* GTP, 2.5 m*M* CTP, 2.5 m*M* UTP, 250 m*M* creatine phosphate, 4 mg/ml creatine kinase, 155 m*M* HEPES–KOH (pH 7.4), 600 m*M* KCH_3CO_2), and 1.25 μg of vRNA. The reactions were incubated at the indicated temperatures for the indicated periods of time.

Viral protein synthesis was assayed by including 1.2 mCi/ml [^{35}S]methionine (1200 Ci/mmol, 15 mCi/ml, Amersham) in the reactions. Samples (1 μl) were removed from the reactions at the indicated times into SDS sample buffer (2% SDS (Sigma), 62.5 m*M* Tris–HCl (pH 6.8), 0.5% 2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol). The proteins were denatured by heating at 100° for 3 min and separated by electrophoresis in a 10% polyacrylamide gel (29:1 acrylamide:bisacrylamide) containing 0.1% SDS, 187.5 m*M* Tris–HCl (pH 8.8) as described (Barton and Flanegan, 1993). The gel was fluorographed using 22% PPO in DMSO, dried, and exposed to Dupont Reflexions Plus film at −70° for 5 hr.

RNA replication was assayed by adding 10 μCi [^{32}P]-

CTP (>400 Ci/mmol, 10 μCi/μl, Amersham) to 50 μl HeLa S10 translation–RNA replication reactions for 1 hr at the indicated times and temperatures. For experiments in which RNA replication was assayed, CTP was not included in the 10× reaction mix indicated above in order to increase the specific activity of the radiolabel. After radiolabeling, the reactions were solubilized by adding 350 μl 0.5% SDS, 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 100 m*M* NaCl. Following phenol:chloroform:isoamyl alcohol extraction (25:24:1) and ethanol precipitation, the RNA products were solubilized in 20 μl of 50 m*M* CH_3HgOH sample buffer (50 m*M* CH_3HgOH, 50 m*M* H_3BO_3, 5 m*M* $Na_2B_4O_{7-}H_2O$, 10 m*M* Na_2SO_4, 1 m*M* Na_2EDTA, pH 8.2) and separated by electrophoresis in a 1% agarose CH_3HgOH gel (Young *et al.,* 1985). The gel was dried and exposed to Dupont Reflexions Plus film and genome-length RNA was quantitated on a PhosphorImager (Molecular Dynamics).

RNA synthesis by preinitiation RNA replication complexes: Guanidine reversal procedure

Preinitiation RNA replication complexes were formed in HeLa S10 translation–replication reactions containing 2 m*M* guanidine HCl and the indicated vRNAs by incubation of the reactions at 34° for 4 hr as described (Barton *et al.,* 1995). The preinitiation complexes were isolated by centrifugation of each 50-μl reaction at 4° for 15 min at 15,000 *g.* For the experiment described in Fig. 6, the supernatants were removed and the pellets containing the preinitiation complexes were resuspended in 50-μl reactions containing 25 μl HeLa S10 extract, 10 μl translation initiation factors, 5 μl 10× nucleotide mix without CTP, and 1 μl [α-^{32}P]CTP (10 μCi/ml, Amersham). For the experiments described in Figs. 7–9, the preinitiation complexes were resuspended in 50-μl reactions containing 35 m*M* HEPES–KOH, pH 7.4, 120 m*M* KCH_3CO_2, 2.75 m*M* $Mg(CH_3CO_2)_2$, 5 m*M* KCl, 3 m*M* DTT, 0.5 m*M* $CaCl_2$, 1 m*M* EGTA, 1 m*M* ATP, 250 μ*M* GTP, 250 μ*M* UTP, 30 m*M* creatine phosphate, 400 μg/ml creatine kinase, the indicated amount of [α-^{32}P]CTP adjusted to a final concentration of 5 μ*M.* The reactions were incubated at the indicated temperatures for the indicated period of time, terminated by the addition of 350 μl 0.5% SDS buffer, phenol:chloroform:IAA extracted, and precipitated with the addition of 3 vol of ethanol and stored at −20° overnight. The RNA was recovered by centrifugation, the ethanol decanted, the RNA dried briefly in vacuo, and solubilized in 20 μl of 50 m*M* CH_3HgOH sample buffer (Young *et al.,* 1985). The RNA samples were separated by electrophoresis in a 1% agarose CH_3HgOH gel and were visualized by autoradiography of the dried gel. RNA present within the gel was quantitated by phosphorimaging (Molecular Dynamics).

RESULTS

RNA synthesis in mutant ts10-infected cells

To characterize the temperature-sensitive RNA-negative phenotype of mutant ts10 (Kuchta *et al.,* 1988), we infected HeLa cells with either ts10 or wild-type virus and measured viral RNA accumulation at the permissive (33°) and nonpermissive (39°) temperatures (Fig. 1). Mutant ts10 and wild-type viruses synthesized similar amounts of RNA at 33° (Fig. 1A). At 39°, however, RNA synthesis in the mutant ts10-infected cells was dramatically reduced relative to the amount of RNA synthesized in cells infected with wild-type virus (Fig. 1B). These results suggested that either a viral protein or a RNA sequence required for RNA replication was altered in mutant ts10.

Characterization of the mutant ts10 polymerase

To determine if the mutant ts10 polymerase was sensitive to heat inactivation *in vitro,* we purified mutant ts10 polymerase from cytoplasmic extracts of HeLa cells infected at 33° for 8 hr. The heat inactivation kinetics for the ts10 polymerase and the wild-type polymerase were determined by incubating the purified enzymes for increasing periods of time at 45° and then determining their residual activities at 25°. The ts10 polymerase was compared to the wild-type polymerase at the Fraction III stage of purification (Young *et al.,* 1986). The mutant ts10 polymerase was rapidly inactivated at 45°, whereas the activity of the wild-type polymerase was only slightly reduced (Fig. 2). This result was observed when either poly(A):oligo(U) or poliovirion RNA:oligo(U) was used as the template:primer pairs in the *in vitro* polymerase assay (Figs. 2A and 2B, respectively). Wild-type polymerase was isolated from cells infected at both 33° for 8 hr and 37° for 5 hr. Both preparations of wild-type polymerase were resistant to heat inactivation at 45° (data not shown). These results indicated that the mutant ts10 $3D^{Pol}$ was heat-labile and suggested that its amino acid sequence was mutated at one or more sites. To identify mutations in ts10 $3D^{Pol}$, we sequenced the $3D^{Pol}$ gene of ts10 virion RNA. A U to C transition was found at nucleotide 7167 (Fig. 3). This mutation resulted in a methionine (AUG codon) to threonine (ACG codon) change at amino acid 394 in mutant ts10 $3D^{Pol}$. No other mutations were identified in the $3D^{Pol}$ gene of ts10.

Engineering the 3D-M394T mutation in a wild-type poliovirus clone

To determine if the 3D-M394T mutation was responsible for the temperature-sensitive RNA-negative phenotype of mutant ts10 virus, we engineered this mutation into an infectious cDNA clone of poliovirus. This mutant (3D-105) was engineered such that the AUG codon for M394 in the wild-type sequence was

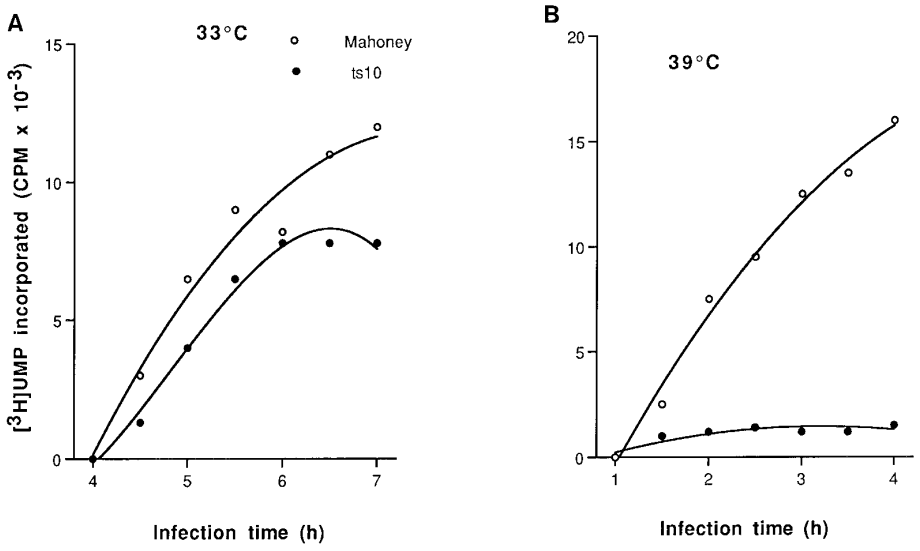

FIG. 1. Poliovirus RNA synthesis is temperature-sensitive in ts10-infected cells. Cells were infected with either wild-type or mutant virus at a m.o.i. of 20 and placed at either 33° (A) or 39° (B). The cultures were treated with actinomycin D (5 μg/ml) at 15 min postinfection and [^{3}H]uridine (5 μCi/ml) was added at 4 hr (A) or 1 hr (B) postinfection. Samples were taken at the indicated times and the amount of [^{3}H]uridine incorporated into RNA was determined as described under Materials and Methods.

changed to ACU (Fig. 3). A double mutation in the nucleotide sequence was used to lower the frequency of reversion. Virus was isolated following transfection of HeLa cells at 32.5° with mutant 3D-105 plasmid DNA (Table 1). Two independent isolates of mutant 3D-105 virus (B-1 and J-12) were characterized in plaque assays at the permissive and nonpermissive temperatures (Table 1). The mutant 3D-105 virus isolates were temperature-sensitive for virus production. Their titers decreased by about 3×10^3 at 39°. The plaques observed at 39° after 2 days of infection with 3D-105 virus were of similar size to those of wild-type Mahoney virus and, therefore, were likely revertants. The decrease in the titer of mutant ts10 virus at 39° was an order of magnitude less than with mutant 3D-105 virus. This may be due to an increased reversion frequency since the ts10 mutant virus has a single-base change relative to wild-type virus in comparison to the two-base change in mutant 3D-105 virus. Interestingly, when plaque assays were incubated for 3 days at 39° rather than 2 days, a large number of minute plaques were present. When these minute plaques were counted, the titers of ts10 and 3D-105 viruses at 39° were very similar to the titers at 32.5°. After 3 days at

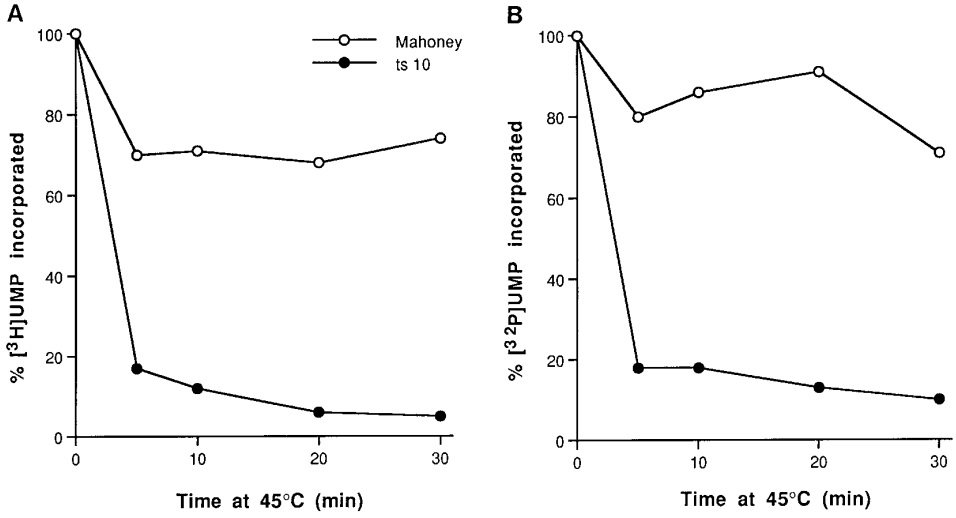

FIG. 2. Heat inactivation of ts10 polymerase activity *in vitro.* (A) Wild-type and ts10 polymerases (2.5 μg Fraction III polymerase) were incubated at 45° for the indicated times and then added to reaction mixtures containing poly(A):oligo(U) and [^{3}H]UTP and were incubated at 25° for 1 hr. (B) The experimental conditions were the same as (A) except the reactions contained poliovirion RNA:oligo(U) and [^{32}P]UTP. The amount of labeled substrate incorporated into product RNA was determined as described under Materials and Methods.

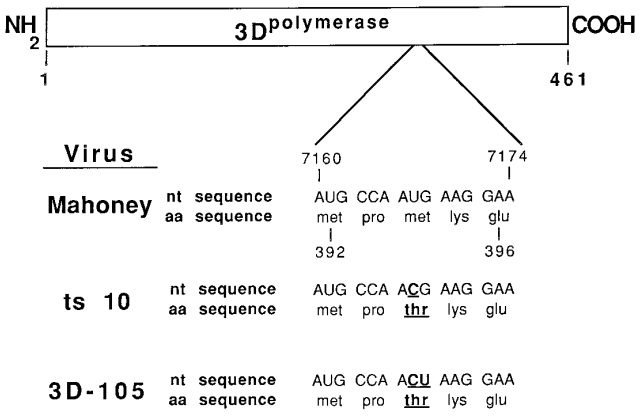

FIG. 3. Poliovirus ts10 and 3D-105 mutations in the $3D^{Pol}$ coding sequence. The complete coding region for the $3D^{Pol}$ gene in ts10 virion RNA was sequenced using the dideoxynucleotide primer-extension method. Ts10 virion RNA contained a C to U transition at nucleotide 7167 resulting in a methionine to threonine change at amino acid 394 of $3D^{Pol}$. Site-directed mutagenesis was used to engineer mutant 3D-105 as described under Materials and Methods. 3D-105 virus contains two nucleotide changes relative to wt Mahoney virus, a U to C transition at nucleotide 7167 and a G to U transversion at nucleotide 7168 resulting in a methionine to threonine change at amino acid 394.

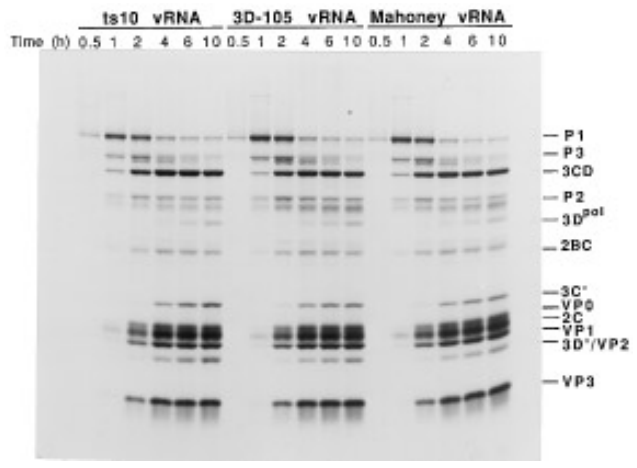

FIG. 4. Translation of ts10, 3D-105, and wt vRNAs *in vitro.* Ts10, 3D-105, and wild-type virion RNAs were added to HeLa S10 *in vitro* translation reactions containing [^{35}S]Met. At the indicated times, samples were removed and fractionated on an SDS–10% polyacrylamide gel as described under Materials and Methods. The gel was fluorographed, dried, and exposed to film.

39°, the ts10 plaques were <1 mm in diameter, whereas the wt Mahoney plaques were >8 mm in diameter (data not shown). Thus, the phenotype of ts10 and 3D-105 viruses could be characterized more accurately as an extreme plaque size mutation at the restrictive temperatures.

Characterization of ts10, 3D-105, and wt Mahoney viruses in HeLa S10 translation–RNA replication reactions

Virion RNAs were isolated from the three viruses and programmed into HeLa S10 translation–RNA replication reactions (Barton *et al.,* 1995). Protein synthesis and polyprotein processing was examined at 33° (Fig. 4). The amount of viral protein synthesized and the kinetics of viral polyprotein processing by the three RNAs were indistinguishable in the *in vitro* reactions (Fig. 4). Protein synthesis was not examined at 39° because the HeLa S10 reactions are incapable of supporting protein synthesis at this temperature (Barton and Flanegan, unpublished observation) (Molla *et al.,* 1993). In this experiment, viral proteins were first synthesized at 30°, and RNA replication was then measured after a shift in the reaction temperature to the values indicated. Mahoney virus RNA synthesis occurred optimally at 37° when the RNA replication complexes were previously formed in reactions at 30° (Fig. 5). Because the Mahoney virus RNA synthesis was not adversely affected at the restrictive temperature *in vitro* (Fig. 5), we examined the optimum temperature for replication of the ts10 and 3D-105 RNAs in the HeLa S10 reactions. The ts10 and 3D-105 RNAs had a clear optimum of 34° for RNA replication in the HeLa S10 reactions (Fig. 5). At the restrictive temperature of 39°, ts10 and 3D-105 virus RNA synthesis was drastically reduced (Fig. 5). This apparent difference in the optimum temperature for RNA replication between the mutant and wild-type viruses in the *in vitro* replication system was consistent with the phenotype of the viruses *in vivo.*

We further characterized the temperature-sensitive defect of mutants ts10 and 3D-105 using temperature-shift experiments in HeLa S10 reactions. At 33°, the permissive temperature, equivalent amounts of the mutant and wild-type RNAs were labeled from 4.5 to 5.5 hr (Fig. 6, lanes 1–3). When identical reactions were incubated at 33° for 4.5 hr, shifted to 39°, and then pulse-labeled for 1 hr, the temperature-sensitive phenotype of the mutant viruses was apparent (Fig. 6, lanes 4–6). Quantitation by phosphorimaging showed that there was a 60% decrease in the replication of the mutant RNAs when compared to

TABLE 1

Plaquing Efficiency of ts10, 3D-105, and Wild-Type Mahoney Viruses under Permissive and Nonpermissive Conditions

	PFU/ml		
Virus	32.5°C	39°C[a]	32.5°C/39°C
wt Mahoney	1.7×10^{10}	2.1×10^{10}	0.8
3D-105 (B-1)	1.3×10^{9}	5.0×10^{5}	2600
3D-105 (J-12)	2.1×10^{9}	5.0×10^{5}	4200
ts10	1.0×10^{9}	7.5×10^{6}	133

[a] Plaques after two days incubation

Note. Plaques of <1 mm in diameter appeared for ts10 at 39° after 3 days at 39° at titers equal to those at 32.5°. Wild-type plaques were >8 mm in diameter after 3 days at 39°.

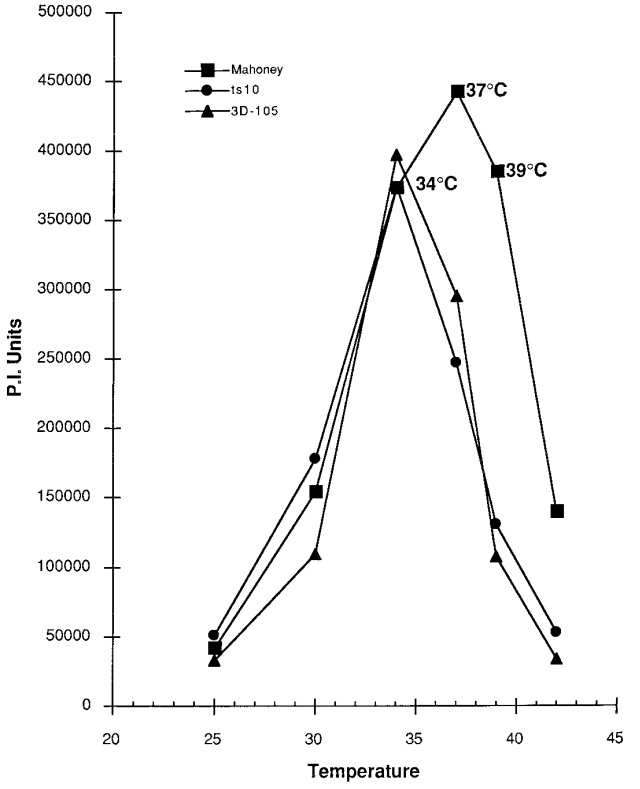

FIG. 5. Determination of optimum temperature for replication of ts10, 3D-105, and wt vRNAs in HeLa S10 translation–RNA replication reactions. Ts10, 3D-105, and wt virion RNAs were added to HeLa S10 *in vitro* translation–RNA replication reactions, and the reactions were incubated at 30° for 5 hr. At 5 hr, [^{32}P]CTP was added to the reactions and they were incubated at the indicated temperatures for 1 hr. The radiolabeled RNA products were fractionated on a denaturing methyl mercury hydroxide 1% agarose gel. The amount of radiolabel in genome-length RNA was quantitated by PhosphoImaging (arbitrary P.I. Units) and plotted versus temperature.

Mahoney RNA (Fig. 6, lanes 4–6). To investigate the effect of the 3D-M394T mutation on the initiation reaction, we used "preinitiation" RNA replication complexes (PI complexes) to assay RNA synthesis at 39° (Fig. 6, lanes 7–9). PI complexes are formed in HeLa S10 translation–RNA replication reactions in the presence of 2 m*M* guanidine–HCl (Barton *et al.,* 1995). The subsequent removal of guanidine from the PI complexes allows for the synchronous initiation of RNA replication (Barton *et al.,* 1995). Using PI complexes formed in reactions containing either ts10, 3D-105, or wild-type RNAs, we assayed for viral RNA synthesis at 39°. The replication of the ts10 and 3D-105 RNAs was inhibited by about 90% compared to the replication wt Mahoney RNA (Fig. 6, lanes 7–9).

Temperature-sensitive defect in 3D-M394T polymerase is not reversible

To determine whether the temperature-sensitive defect in RNA replication exhibited by the ts10 and 3D-105 PI RNA replication complexes was reversible, we assayed RNA replication within PI RNA replication complexes at 34° after a 30-min preincubation at 39.5° (Fig. 7). After the shift-up and shift-down in temperature, the 3D-105 PI replication complexes synthesized very small amounts of labeled viral RNA at 34° (Fig. 7, lane 3) as compared to 3D-105 PI replication complexes maintained at 34° (Fig. 7, lane 2). In a shift-up experiment where RNA synthesis was measured at 39.5° after a 30-min preincubation at this temperature, no labeled viral RNA was detected (Fig. 7, lane 4). Control reactions containing wt Mahoney RNA were unaffected by preincubation at 39.5° (Fig. 7, lanes 6–8). Thus, the temperature-sensitive defect exhibited by 3D-105 PI replication complexes at 39.5° was not reversible upon shifting back to 34°, the permissive temperature.

We next examined the kinetics of heat-inactivation within PI replication complexes and determined if the 3D-M394T mutation affected negative-strand RNA synthesis. In order to assay negative-strand RNA synthesis, PI replication complexes were incubated in reactions containing 5 μM CTP at 34° for 20 min (Fig. 8). Under these conditions, PI replication complexes synchronously initiate and synthesize full-length negative-

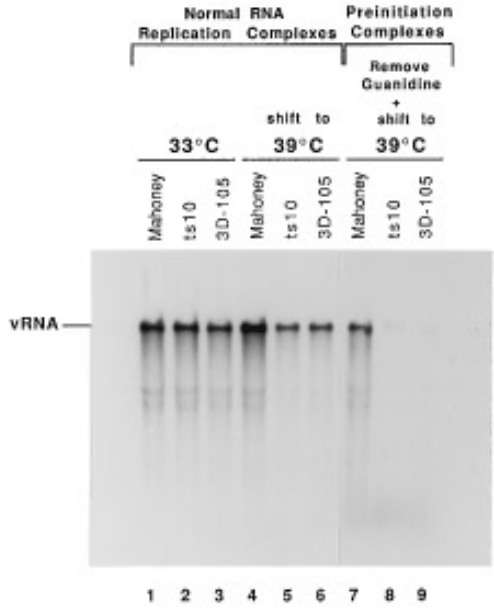

FIG. 6. Replication of ts10, 3D-105, and wt RNAs in HeLa S10 reactions and preinitiation RNA replication complexes at 33 and 39°. Wt Mahoney, ts10, and 3D-105 virion RNAs were added to HeLa S10 translation–RNA replication reactions. The reactions were then incubated at 33° for 4.5 hr in the absence of guanidine HCl (lanes 1–6) or in the presence of 2 m*M* guanidine HCl (lanes 7–9). [^{32}P]CTP was added to the reactions without guanidine HCl and they were incubated at 33° for 1 hr (lanes 1–3) or 39° for 1 hr (lanes 4–6). Preinitiation replication complexes were isolated by centrifugation from the reactions containing guanidine HCl and resuspended in fresh reaction mixtures containing [^{32}P]CTP and incubated at 39° for 1 hr (lanes 7–9). The radiolabeled RNA products were fractionated on a denaturing CH_3HgOH 1% agarose gel. The gel was dried and exposed to film.

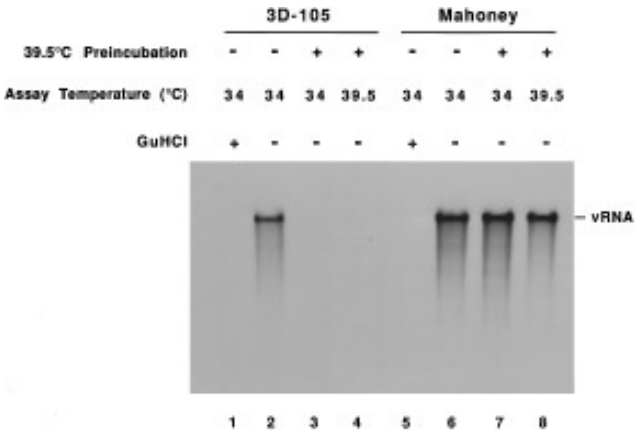

FIG. 7. Irreversible heat-inactivation of 3D-105 preinitiation RNA replication complexes. 3D-105 and wt Mahoney RNAs were added to HeLa S10 translation-replication reactions containing 2 m*M* guanidine HCl and incubated at 34° for 4 hr to form preinitiation RNA replication complexes. The reactions were then preincubated for 30 min at 34° (lanes 1, 2, 5, 6) or 30 min at 39.5° (lanes 3, 4, 7, 8). Preinitiation replication complexes were isolated by centrifugation at 4° from the reactions containing guanidine HCl and were resuspended in new reaction mixtures containing 20 μCi [^{32}P]CTP. The reactions were then incubated for 1 hr at 34° (lanes 1, 2, 3, 5, 6, 7) or 39.5° (lanes 5 and 8). 2 m*M* guanidine was included in two reactions (lanes 1 and 5). The radiolabeled RNAs were separated by electrophoresis on a CH_3HgOH-1% agarose gel and the dried gel was subjected to autoradiography.

strands. Positive-strand RNA synthesis initiates, but full-length positive-strands are not synthesized because the time of incubation is insufficient (D. J. Barton and J. B. Flanegan, unpublished observations). Both 3D-105 and wt Mahoney PI replication complexes synthesized similar amounts of negative-strand RNA at 34° (Fig. 8, lanes 2 and 7). When 3D-105 PI replication complexes were preincubated at 39.5° for increasing periods of time, negative-strand RNA synthesis was increasingly inhibited when assayed at 34° (Fig. 8, lanes 3–5). The heat inactivation of 3D-105 PI replication complexes was rapid, with greater than 95% inactivation after 10 min at 39.5° (Fig. 8, lane 4). In contrast, wt Mahoney PI replication complexes were unaffected for negative-strand synthesis after preincubation at 39.5° (Fig. 8, lanes 7–10). The results showed that negative-strand RNA synthesis in 3D-105 replication complexes was rapidly and irreversibly heat-inactivated at 39.5°.

The heat-inactivation of negative-strand synthesis in 3D-105 replication complexes may result from an inhibition in the initiation step, the elongation step, or both. To differentiate between these possibilities, we used a time-course experiment to directly measure the elongation rate in 3D-105 RNA and wt RNA replication complexes at 39.5°. In this experiment, the PI replication complexes were formed at 34° in HeLa S10 translation reactions containing guanidine and the PI replication complexes were then isolated by centrifugation. The time-course reactions were started by resuspending the PI replication complexes in assay reaction mixtures containing 5 μM [^{32}P]CTP and were incubated from 6 min to 16 min at 39.5°. The specific radioactivity of the [^{32}P]CTP was increased fivefold in the 3D-105 reaction to normalize the amount of labeled RNA synthesized at each time point in both reactions. The results showed that the nascent negative-strands of both the 3D-105 RNA and the wt RNA were about the same size at 6 and 8 min and that both were full-length by 10 min (Fig. 9). Therefore, the estimated chain elongation rate of 800 nucleotides/min was identical in both reactions. These results indicate that the heat-sensitive step in negative-strand RNA synthesis exhibited by 3D-105 replication complexes is the initiation of RNA synthesis and not in the elongation of nascent chains.

DISCUSSION

In this study we characterized the temperature-sensitive RNA-negative phenotype of poliovirus mutant ts10 (Hewlett *et al.,* 1982). Using purified polymerase from infected cells, we showed that ts10 polymerase activity was rapidly and irreversibly inactivated at 45° *in vitro* while the wild-type polymerase was relatively stable to heat inactivation. This suggested that the ts10 polymerase was mutated at one or more sites. We sequenced the 3D^{Pol} gene of ts10 virion RNA and identified a single point mutation, a U to C transition at nucleotide 7167,

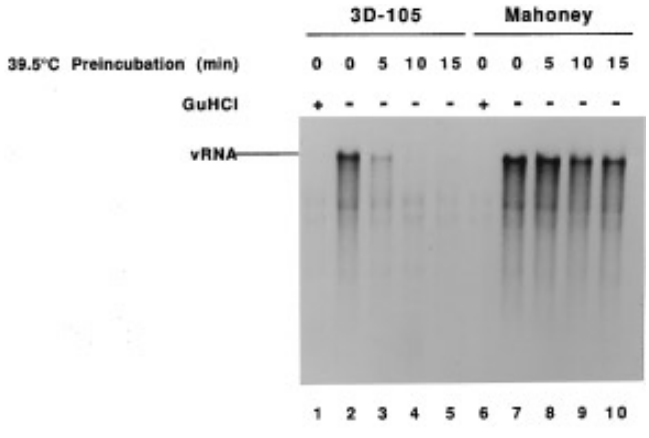

FIG. 8. Heat inactivation of negative-strand RNA synthesis in 3D-105 preinitiation RNA replication complexes. 3D-105 and wt Mahoney RNAs were added to HeLa S10 translation-replication reactions containing 2 m*M* guanidine HCl and incubated at 34° for 4 hr to form preinitiation RNA replication complexes. The reactions were then incubated a total of 15 min at 34 or 39.5° as follows: 15 min at 34° (lanes 1, 2, 6, and 7), 10 min at 34°, and then 5 min at 39.5° (lanes 3 and 8), 5 min at 34°, and then 10 min at 39.5° (lanes 4 and 9), or 15 min at 39.5° (lanes 5 and 10). For clarity, the times incubated at 39.5° for each lane are as described in the legend of Fig. 7. Preinitiation complexes were then recovered by centrifugation at 4° and resuspended in reactions containing 20 μCi [^{32}P]CTP and incubated at 34° for 20 min. 2 m*M* guanidine HCl was added to two reactions (lanes 1 and 6). The radiolabeled products were analyzed by electrophoresis and autoradiography as described under Materials and Methods.

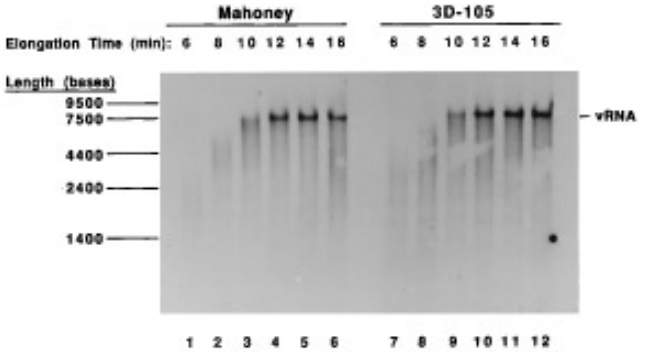

FIG. 9. Elongation of negative-strand RNA within 3D-105 RNA replication complexes is not inhibited at 39.5°. 3D-105 and wt Mahoney RNAs were added to HeLa S10 translation-replication reactions containing 2 m*M* guanidine HCl and incubated at 34° for 4 hr to form preinitiation RNA replication complexes. The preinitiation RNA replication complexes were isolated by centrifugation and resuspended in 50-μl reactions containing 50 μCi [^{32}P]CTP or 10 μCi [^{32}P]CTP for the mutant and wt replication complexes, respectively. The reactions were incubated for the indicated periods of time at 39.5°. The radiolabeled RNAs were separated by electrophoresis on a CH_3HgOH-1% agarose gel and the dried gel was subjected to autoradiography.

which resulted in a methionine to threonine substitution at amino acid 394 of the 3D^{Pol} protein. The 3D-M394T mutation was engineered into an infectious poliovirus cDNA clone. The virus produced from this mutant clone, 3D-105 virus, had a temperature-sensitive RNA-negative phenotype identical to ts10 virus. Further characterization of the ts10 and 3D-105 mutants in HeLa S10 RNA replication reactions demonstrated that the 3D-M394T mutation resulted in a severe defect at the initiation step of viral RNA replication.

Clustered charged-to-alanine mutagenesis of charged amino acids in the region of 3D^{Pol} containing the M394T mutation were all lethal (Diamond and Kirkegaard, 1994) (Fig. 10). Six such lethal mutations mapped within a 39 amino acid long region (K375 to H413) immediately C-terminal of the putative polymerase core domain (Diamond and Kirkegaard, 1994) (Fig. 10). Computer alignments of this region of 3D^{Pol} in the human enteroviruses indicate that the amino acid sequence in this region is very highly conserved (Ann Palmenberg, personal communication) (Fig. 10). In each of the lethal charged-to-alanine mutations, one or more of the conserved amino acids in this region was changed (Fig. 10). As previously discussed by Diamond and Kirkegaard (1994), the disruption of clusters of charged amino acids, which are expected to be on the solvent-exposed surface, may disrupt the interaction of the polymerase with other macromolecules or with the solvent. Therefore, this group of lethal clustered charged-to-alanine mutations from K375 to H413 may define, at least in part, a solvent-exposed surface region of 3D^{Pol}. The M394T mutation examined in this study is in the center of this region (Fig. 10).

HeLa S10 translation–replication reactions were shown previously to efficiently support poliovirus replication only at temperatures of 34° or lower (Molla *et al.,* 1993). At higher temperatures, viral polyprotein processing was adversely affected (Molla *et al.,* 1993) and overall protein synthesis was drastically reduced (D. J. Barton and J. B. Flanegan, unpublished observation). Despite these limitations, we found that poliovirus RNA replication proceeded efficiently at 37–39° for wild-type Mahoney virus upon shift-up of HeLa S10 translation–replication reactions incubated at 34°. The methodology developed in this study allows for the formation of normal RNA replication complexes or preinitiation RNA replication complexes (replication complexes formed in the presence of 2 m*M* guanidine HCl) at temperatures permissive for protein synthesis and polyprotein processing (34°) followed by shifting to temperatures optimal for RNA replication. The 3D-M394T mutation caused a clear shift in the optimum temperature for viral RNA replication from 37 to 34°. Poliovirus mutants ts10 and 3D-105 were clearly temperature-sensitive for RNA replication in this *in vitro* system immediately upon shift-up to the nonpermissive temperature, consistent with the temperature-sensitive phenotype of both mutants *in vivo.*

The reduced level of mutant RNA synthesis observed at 39° indicated that either the polymerase initiation or elongation reactions (or both) were inhibited. RNA replication complexes, actively engaged in the initiation and elongation of viral RNA molecules, are formed in HeLa S10 translation–replication reactions by 4 hr at 34° (Barton *et al.,* 1995). Mutant RNA replication complexes synthesized 60% less viral RNA in a 1 hr pulse-label when shifted to 39° as compared to Mahoney RNA replication complexes. A complete block in the elongation reaction should inhibit the elongation of nascent RNA molecules and the synthesis of any labeled full-length RNA. Our results showed that the synthesis of labeled full-length RNA was reduced, but not completely blocked in the mutant RNA replication complexes at 39°. Therefore, these results suggested that the elongation reaction was at most only partially inhibited within the mutant replication complexes at 39°. In subsequent experiments, we showed that the 3D-105 polymerase was able to maintain an elongation rate equal to that of the wt polymerase for 10 min or more at the restrictive temperature. Based on these results, we concluded that polymerase already engaged with a template and actively elongating a nascent RNA strand was resistant to heat-inactivation. Because the 3D-M394T mutation did not affect the elongation rate adversely at 39.5°, it implies that a defect exists in RNA synthesis initiation.

To directly measure the effect of the 3D-M394T mutation on the initiation of negative-strand RNA synthesis, we used PI replication complexes. Preinitiation RNA replication complexes must initiate RNA replication upon their incubation in reactions lacking guanidine HCl, first initiating and synthesizing genome length negative-

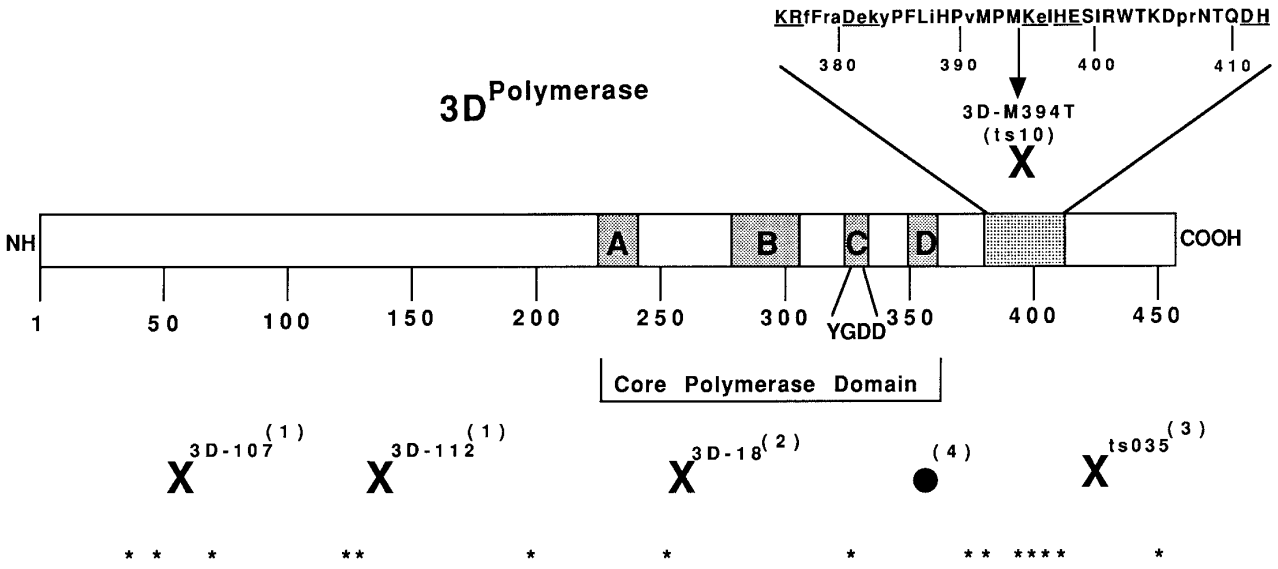

FIG. 10. Map of mutations in $3D^{Pol}$ protein sequence. The diagram depicts the linear sequence of the $3D^{Pol}$ protein and indicates the location of many relevant mutations characterized in the literature. Large X, conditional lethal temperature-sensitive mutants (Diamond and Kirkegaard, 1994 (1); Charini *et al.* 1991 (2); Agut *et al.* 1989 (3)); *, clustered charged-to-alanine lethal mutations (Diamond and Kirkegaard, 1994); filled circle, small plaque insertion mutation, noncomplementable (Bernstein *et al.,* 1986) (4). The $3D^{Pol}$ amino acid sequence from K375 to H413 is expanded above the diagram. Amino acids which are absolutely conserved in computer alignments of the human enteroviruses are shown in capital letters (A. Palmenberg, personal communication). Underlined amino acids indicate the sites of five different clustered charged-to-alanine lethal mutants previously described (Diamond and Kirkegaard, 1994). The position of the 3D-M394T mutation is indicated by the arrow.

strand RNAs followed by the initiation and elongation of positive-strand RNAs (Barton *et al.,* 1995). We found that the ts10 and 3D-105 mutant preinitiation complexes were dramatically inhibited in their ability to initiate negative-strand RNA synthesis at the nonpermissive temperature. The irreversible nature of the temperature-sensitive defect of the 3D-105 mutant suggests that a significant alteration occurs within $3D^{Pol}$ at 39.5° in the RNA replication complex. The irreversible inactivation of $3D^{Pol}$ (or a $3D^{Pol}$ precursor protein, e.g., 3CD) may prevent its association with either the template RNA or with viral or cellular proteins that are required for the initiation of RNA replication. In future studies, it will be important to determine if the 3D-M394T mutation also blocks the initiation of positive-strand RNA synthesis and to further characterize the molecular basis for the inhibition of the initiation reaction by this mutation.

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