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Temperature-sensitive RNA polymerase mutants of a picornavirus

(foot-and-mouth disease virus/conditional-lethal isoelectric focusing/reversion/thermal inactivation of transcription)

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ABSTRACT **Temperature-sensitive (*ts*) RNA polymerase mutants of a picornavirus are reported. Two foot-and-mouth disease virus (FMDV) mutants designated *ts* 22 and *ts* 115 have been characterized. As judged by isoelectric focusing, both have charge alterations in P56a, the FMDV RNA polymerase protein. Virus RNA synthesis in cells infected with the mutants is severely impaired at the nonpermissive temperature. RNA polymerase purified from baby hamster kidney cells infected with these mutants exhibits a marked *ts* transcribing activity *in vitro*. Spontaneous revertants of both mutants have P56a polypeptides that are indistinguishable from the parental proteins on the basis of charge. The revertants regain the ability to synthesize virus RNA *in vivo* at the nonpermissive temperature. RNA polymerase purified from the revertants remains transcriptionally active at the nonpermissive temperature.**

The genome of foot-and-mouth disease virus (FMDV) is a single-stranded "positive"-sense RNA molecule of approximately 8 kilobases with a small protein termed VPg covalently attached to its 5′ end, an internal poly(C) tract, and a poly(A) sequence at the 3′ end (1) (Fig. 1). Replication of the virus RNA involves the synthesis of complementary negative strands followed by transcription of positive RNA molecules with the negative strands as template (2). A nonstructural virus-induced polypeptide, P56a, has been identified as the enzyme responsible for FMDV RNA chain elongation *in vivo* (3–5). FMDV RNA polymerase has been purified from the cytoplasm of infected cells and shown to contain the virus protein P56a with trace amounts of its transcriptionally inactive precursor P72 (4). Purified FMDV RNA polymerase is capable of initiating RNA synthesis *in vitro* on an FMDV RNA template. The enzyme also transcribes *in vitro* the synthetic template poly(A) primed with $(Up)_5U$ with a poly(U) polymerase activity (6). Biochemical mapping of FMDV-coded proteins with pactamycin has shown that P56a is encoded by a region close to the 3′ end of the genome (7, 8). It is not yet known whether host-coded proteins are involved in FMDV RNA synthesis.

Conditional-lethal mutants of several picornaviruses have been reported that have defects in the level or type of RNA synthesized *in vivo* at the nonpermissive temperature (9, 10). The present studies differ from this previous work in two important respects. First, the location of the *ts* mutants has been correlated with a specific virus-coded polypeptide. Second, it has been demonstrated that this isolated polypeptide from such mutants possesses a *ts* transcribing activity *in vitro*.

Seventy spontaneous or chemically induced *ts* FMDV mutants have been isolated and mapped by recombination (11–13). Electrofocusing (EF) has been used to identify alterations in FMDV structural proteins that result from *ts* mutations of the FMDV genome (14, 15). Hence, a tentative alignment of the genetic and biochemical maps has been constructed (15). We describe how a modified EF technique was used to screen mutants with *ts* loci at the 3′ end of the FMDV genetic map for an altered P56a polypeptide. Two candidate RNA polymerase mutants (*ts* 22 and *ts* 115) were identified by these criteria. Cells infected with these mutants synthesize only trace amounts of virus RNA at the nonpermissive temperature. Furthermore, FMDV RNA polymerase purified from the cytoplasm of baby hamster kidney cells infected with the mutants showed a *ts* transcribing activity *in vitro* when compared with the enzyme extracted from cells infected with the parental virus.

MATERIALS AND METHODS

Viruses. The parental FMDV strain from which all mutants were derived was O Pacheco (ts^+). Mutants mapping at the 3′ end of the FMDV genome (5-fluorouracil-induced *ts* 22 and spontaneous *ts* 115) have been described (12, 13). The isolation of a revertant of *ts* 22 (designated *ts* 22R) also has been described (15). The spontaneous revertant of *ts* 115, termed *ts* 115R, was obtained in an identical manner. A spontaneously occurring VPI mutant, *ts* 103 (15), was also examined. Virus was propagated by using monolayers of baby hamster kidney 21 C13 cells at the permissive temperature (37°C) or nonpermissive temperature (41°C).

Assay of FMDV Poly(U) and FMDV RNA-Dependent RNA Polymerase. FMDV poly(U) polymerase was assayed *in vitro* by measuring the incorporation of [^{3}H]UTP into poly(U) with poly(A) as template and $(Up)_5U$ as primer (4, 6). FMDV RNA-dependent RNA polymerase was assayed *in vitro* by measuring the incorporation of [^{3}H]CTP into RNA in the presence of all four ribonucleoside triphosphates, an FMDV (serotype O) RNA template, and $(Up)_5U$ primer (4). Assay temperatures corresponding to permissive or nonpermissive conditions are shown on the appropriate figures.

Purification of FMDV RNA Polymerase. The enzyme was isolated from the cytoplasm of approximately 5×10^8 infected cells by DEAE-cellulose and poly(U)-Sepharose chromatography (4). $NaDodSO_4$/polyacrylamide gel analysis (16) of the virus-induced methionine-labeled polypeptides showed that the purified enzyme consisted of P56a with traces of its transcriptionally inactive precursor P72. To screen a relatively large number of *ts* mutants and revertants simultaneously, a related rapid small-scale purification procedure was developed. Approximately 10^8 methionine-labeled infected cells were grown and washed as described (4). The cells were homogenized in 2.5 ml of 0.01 M Tris·HCl, pH 8/0.01 M NaCl and centrifuged for 15 min in an Eppendorf microcentrifuge at 4°C. Each supernatant was mixed with an equal volume of buffer A [0.05 M Tris·HCl, pH 8/0.1 mM dithiothreitol/0.5% Nonidet P-40/20% (vol/vol) glycerol] and loaded at 4°C onto a 0.7×4 cm Bio-Rad Econocolumn containing 1 ml of DE-52 (Whatman) DEAE-cellulose previously washed (4) and equilibrated in buf-

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Abbreviations: EF, electrofocusing; IEF, isoelectric focusing; FMDV, foot-and-mouth disease virus; ts, temperature sensitive.

fer A. After washing the column with 10 ml of buffer A containing 0.05 M KCl, the enzyme was eluted in a minimal volume (approximately 1 ml) of buffer A containing 0.65 M KCl and either diluted 1:10 in buffer A or dialyzed overnight against 2 liters of buffer A. The enzyme was applied to an identical column containing 1 ml of poly(U)-Sepharose (Pharmacia) previously washed (4) and equilibrated in buffer A. The column was washed with 10 ml of buffer A containing 0.05 M KCl, and the RNA polymerase was eluted in a minimal volume of buffer A containing 0.5 M KCl. The purified enzyme was dialyzed against buffer A and stored at −20°C. FMDV RNA polymerase isolated in this way was shown by $NaDodSO_4$/polyacrylamide gel electrophoresis to consist of P56a with low levels of P72, P38, VP3, and contaminating cellular proteins (data not shown).

RESULTS

Identification of Altered P56a Polypeptides in Extracts of FMDV-Infected Cells. Baby hamster kidney cells infected with FMDV synthesize viral proteins that are cleaved to the final products shown in Fig. 1. Isoelectric focusing (IEF) of infected cell cytoplasm in the first dimension, followed by $NaDodSO_4$/polyacrylamide gel electrophoresis in the second dimension, displays many of the FMDV-induced polypeptides (Fig. 2*A*). The pattern obtained from the parental virus ts^+ is shown. Each polypeptide is represented by a single major spot with the exception of P72, which invariably appeared as a smeared double spot. A two-dimensional analysis of a mixture of ts^+- and *ts* 115-induced proteins is shown in Fig. 2*B*. All the proteins appeared as single spots except for P56a and P72, which were represented as double spots. The P56a protein from *ts* 115 had an increased negative charge compared with the P56a from ts^+. P72, the precursor of P56a, also exhibited a similar charge alteration in *ts* 115. In this case, the altered P72 polypeptide (Fig. 2*B*, arrow) is superimposed over the minor spot seen in the P72 of ts^+ (Fig. 2*A*). An identical two-dimensional analysis was performed on a mixture of ts^+- and *ts* 22-induced proteins (data not shown). In this case, charge shifts were detected in both P56a and VP2. The P56a and VP2 proteins from *ts* 22 had decreased and increased negative charges, respectively, when compared with these proteins from ts^+. It is not possible to display all FMDV-induced proteins by a single IEF–$NaDodSO_4$/polyacrylamide gel analysis (14). Full details of the various labeling and EF/IEF conditions required to accomplish this will be published elsewhere. Analysis of all the induced proteins of *ts* 22 and *ts* 115 failed to detect alterations in any other polypeptides (data not shown). Chemically induced *ts* 22 must contain at least two mutations because both VP2 and P56a exhibited altered polypeptides. However, previous EF studies on revertants of *ts* 22 have shown that the charge alteration in VP2 is not correlated with the *ts* defect (15). Hence, on the basis of their genetic map position (13) and alteration in their P56a polypeptides, *ts* 22 and *ts* 115 were considered to be candidate FMDV RNA polymerase mutants.

EF of FMDV RNA Polymerase Purified from *ts* 22- and *ts* 115-Infected Cells. Single-dimension EF gels of purified *ts* 22 and *ts* 115 RNA polymerase (Fig. 3) confirmed the results of the two-dimensional analysis of the total cytoplasmic extract (Fig. 2 *A* and *B*). The induced polypeptide P56a of both mutant enzymes exhibited the previously noted relative charge changes with respect to ts^+ RNA polymerase. Purified P56a from cells infected with the spontaneous revertants of *ts* 22 and *ts* 115 (*ts* 22R and *ts* 115R) were indistinguishable from each other and from ts^+ RNA polymerase (Fig. 3). This shows that the *ts* defects of *ts* 22 and *ts* 115 are both correlated with charge alterations in P56a. The enzyme isolated from cells infected with the VP1 mutant *ts* 103 was indistinguishable from ts^+ RNA polymerase (data not shown).

***In Vivo* FMDV RNA Synthesis in Cells Infected with *ts* 22 and *ts* 115.** The level of FMDV RNA synthesis at the permissive temperature in cells infected with *ts* 22, *ts* 115, *ts* 22R, *ts* 115R, and *ts* 103 is compared with that obtained from ts^+-infected cells in Fig. 4. In all cases, greater than 50% of the ts^+ control activity was observed. However, at the nonpermissive temperature, cells infected with *ts* 22 and *ts* 115 synthesized only trace amounts of virus RNA. Cells infected with *ts* 22R, *ts* 115R, and *ts* 103 retained over 50% of their synthetic activity at the nonpermissive temperature. The inability of the *ts* mutants to synthesize virus RNA at the nonpermissive temperature is consistent with their possessing *ts* RNA polymerases.

Determination of virus RNA synthesis levels in infected cells at the permissive and nonpermissive temperatures is a useful rapid screening procedure for putative RNA polymerase mutants. However, it should be emphasised that this evidence alone is an insufficient diagnostic test because it is possible that such mutants may be defective in an unrelated function that only indirectly affects RNA synthesis. The temperature sensitivity of FMDV RNA polymerase purified from *ts* 22- and *ts* 115-infected cells was therefore examined *in vitro*.

***In Vitro* Transcribing Activity of FMDV RNA Polymerase Purified from *ts* 22- and *ts* 115-Infected Cells.** FMDV RNA polymerase isolated from cells infected with ts^+, *ts* 22, *ts* 115, *ts* 22R, *ts* 115R, or *ts* 103 exhibited a linear poly(U) polymerase activity at 33°C (Fig. 5*A*). Both parental, revertant, and *ts* 103 mutant enzymes retained this activity for up to approximately 40 min at 41°C. The fact that the poly(U) polymerase of the VP1

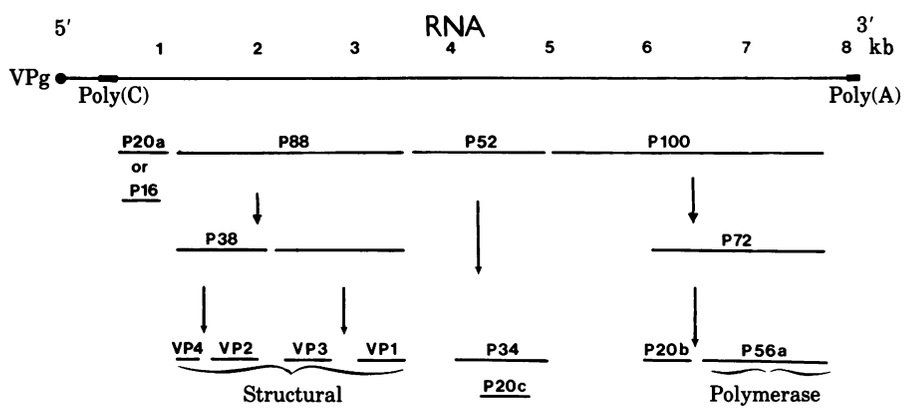

FIG. 1. Biochemical map of the FMDV genome. The RNA molecule is drawn to scale, and cleavage pathways (1) are represented in a simplified form. An alternative cleavage of P100 to P56c and P56a is not shown.

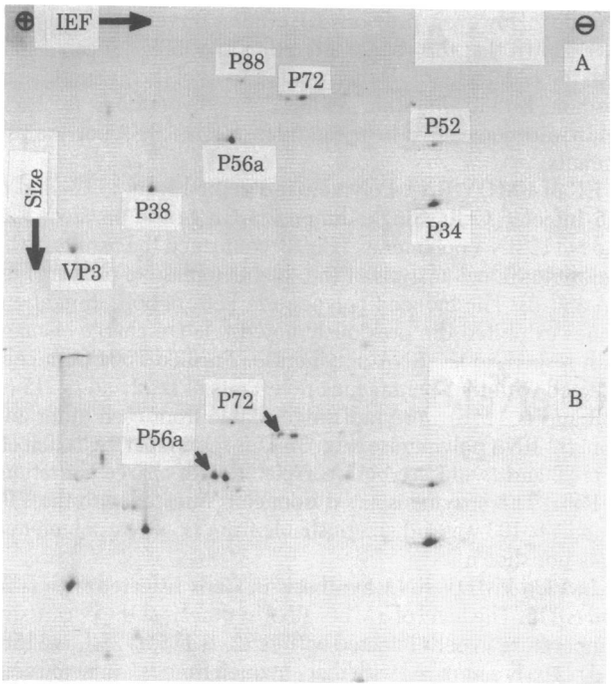

FIG. 2. Two-dimensional IEF–$NaDodSO_4$/polyacrylamide gel electrophoresis analysis of FMDV-induced polypeptides. Approximately 5×10^5 cells in a 2-cm^2 well of a tissue culture plate (Linbro) were infected with FMDV at a multiplicity of infection of approximately 100 at 37°C. After 30 min, the medium was replaced with methionine-free Eagle's medium containing 40 mM Hepes and replaced three more times at 30-min intervals. At 150 min after infection, the cells were labeled with 50 μCi (1 Ci = 3.7×10^{10} becquerels) [^{35}S]methionine in 0.1 ml of methionine-free Eagle's medium for 10 min. The plate was cooled on ice, and the cells were washed once in methionine-free Eagle's medium prior to lysis in 0.1 ml of 0.01 M Tris·HCl, pH 7.4/1 mM EDTA/1% Nonidet P-40/0.1 mg of pancreatic ribonuclease per ml. A cytoplasmic extract was prepared by centrifuging the lysate at 2500 rpm for 10 min and incubating the supernatant for 15 min at 37°C. 2-Mercaptoethanol and Nonidet P-40 were added to give final concentrations of 3.5% (vol/vol) each, followed by urea (Aristar, British Drug House, Poole, England) to a final concentration of 9 M. Methods of IEF (17) were as described (14) with the modifications stated here. IEF gels contained 2% (vol/vol) Nonidet P-40 and a 1:1 (vol/vol) mixture of pH 5–7 and pH 7–9 Ampholines (LKB). Before electrophoresis, cytoplasmic extracts were mixed with unlabeled FMDV structural polypeptides (type O). These proteins eventually served as Coomassie blue-stained markers for the alignment of the gels for autoradiography. The upper (anodic) solution was 0.01 M H_3PO_4 and the lower (cathodic) solution was 0.02 M NaOH. Electrophoresis was for 1 hr at 200 V, 16 hr at 400 V, and 1 hr at 800 V. The second dimension [$NaDodSO_4$/10% (wt/vol) polyacrylamide gel electrophoresis] was performed as described (18). (*A*) Pattern from a cytoplasmic extract of ts^+-infected cells. (*B*) Pattern from a mixture of cytoplasmic extracts from cells separately infected with ts^+ and *ts* 115. The identity of a number of induced polypeptides is given in *A*. Arrows in *B* indicate *ts* 115 polypeptides which differ in charge from their ts^+ counterparts.

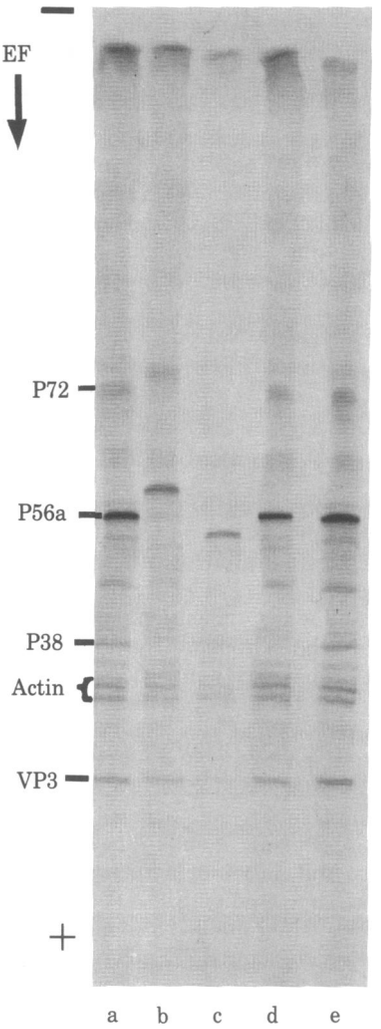

FIG. 3. Single-dimension EF gels of purified FMDV RNA polymerase. $NaDodSO_4$ and 2-mercaptoethanol were added to purified FMDV RNA polymerase in buffer A to give final concentrations of 0.5% and 2% (vol/vol), respectively, and the mixture was heated at 100°C for 1 min. Samples of up to 50 μl were mixed with unlabeled FMDV structural polypeptide markers and were examined by EF (18) with method *ii* described in ref. 14. Gels: RNA polymerase isolated by the small-scale purification procedure from *ts* 115R (a); *ts* 22 (b); *ts* 115 (c); *ts* 22R (d), and ts^+ (e). This analysis was repeated on each of the above samples mixed with ts^+ RNA polymerase. P56a from each of *ts* 22R, *ts* 115R, and *ts* 103 was found to comigrate with the P56a of ts^+ (data not shown).

mutant, *ts* 103, was active at the nonpermissive temperature shows that the inability of *ts* mutants to grow at 41°C is not necessarily caused by a primary defect in RNA replication. In contrast, the poly(U) polymerase activity of the candidate RNA polymerase mutants, *ts* 22 and *ts* 115, was severely impaired at 41°C. To eliminate the possibility that *ts* RNA polymerase preparations contained heat-activated inhibitory impurities, mixtures of ts^+ and *ts* RNA polymerase preparations were assayed for poly(U) polymerase activity at 33°C and 41°C. The mixtures showed additive activities at 33°C and intermediate inhibition at 41°C, indicating that the observed inhibition at 41°C was not due to inhibitory impurities (data not shown).

The ability of ts^+ and *ts* 115 RNA polymerase to transcribe an FMDV RNA template *in vitro* at the permissive and nonpermissive temperatures was investigated (Fig. 5*B*). Both ts^+ and *ts* 115 enzymes exhibited linear RNA synthesis at 33°C. As expected, *ts* 115 RNA polymerase activity was inhibited at the nonpermissive temperature. However, a temperature of 44.5°C was required to inhibit the *ts* 115 FMDV RNA-dependent RNA polymerase, whereas a temperature of only 41°C was needed to inhibit *ts* 115 poly(U) polymerase activity (Fig. 5*A*). It may be that the enzyme is stabilized by FMDV RNA or by the additional nucleoside triphosphate present in the FMDV RNA-dependent RNA polymerase assay.

DISCUSSION

The approach of screening *ts* mutants with loci at the 3′ end of the FMDV genetic map for altered P56a polypeptides and an

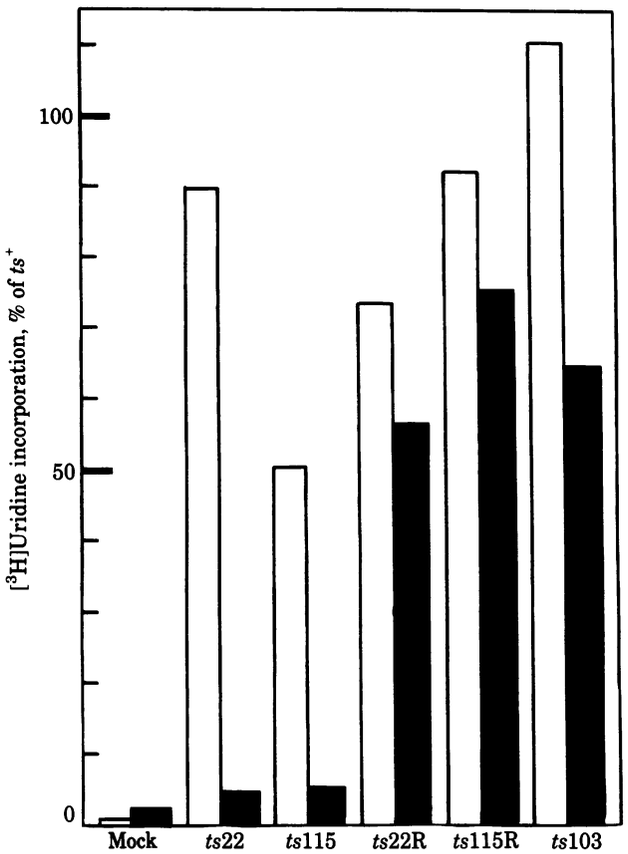

FIG. 4. *In vivo* levels of FMDV RNA synthesis at the permissive and nonpermissive temperatures. FMDV RNA synthesis was measured by [^{3}H]uridine incorporation of infected-cell monolayers in the presence of actinomycin D (Sigma). Cell monolayers were propagated on the bottom of plastic tissue culture test tubes (1.2-cm internal diameter). Duplicate cultures of approximately 2×10^5 cells were infected with FMDV at a multiplicity of infection of approximately 100 at 36.6°C. After 30 min, the medium was replaced with 1 ml of Hepes-buffered Eagle's medium containing 1 μg of actinomycin D per ml. One of each pair of cultures was shifted to 41°C (■), and the other was kept at 36.6°C (□). Uridine incorporation was measured at the previously determined peak of FMDV RNA synthesis in the growth cycle of ts^+. This occurred at 120 min and 150 min after infection for the cultures at 41°C and 36.6°C, respectively. At these times, the medium was replaced with 0.3 ml containing 2 μCi of [^{3}H]uridine (Amersham, 30 Ci/mmol). After 15 min, the cultures were cooled on ice, the medium was removed, and 2.5 ml of cold 5% (wt/vol) trichloracetic acid was added. The samples were centrifuged at 4000 rpm for 10 min, and the pellet was washed twice with 5% trichloracetic acid. Pellets were dissolved in 50 μl of formic acid, and the solution was spotted onto GF/A discs (2.3-cm diameter), dried, and assayed for radioactivity. Cells infected with ts^+ virus incorporated 28,874 cpm at 36.6°C and 12,148 cpm at 41°C. The [^{3}H]uridine incorporation of the viruses indicated in the figure is expressed as a percentage of these levels. Mock-infected cells showed the indicated low levels of [^{3}H]uridine incorporation in the presence of actinomycin D.

inability of the purified enzyme to transcribe at 41°C has led to the identification of novel picornavirus RNA polymerase mutants. These findings support the tentative alignment of the biochemical and genetic map that has been proposed (15). The spontaneous occurrence and reversion of *ts* 115 taken together with the behavior of mutant and revertant P56a polypeptides on IEF gels suggest that this particular mutant is the result of a single base change in the P56a gene. Furthermore, none of the parental, mutant, or revertant P56a polypeptides were separable by $NaDodSO_4$/polyacrylamide gel electrophoresis (data not shown). Several mechanisms for *ts* 115 reversion are possible. First, the parental base could be regenerated. Substitu-

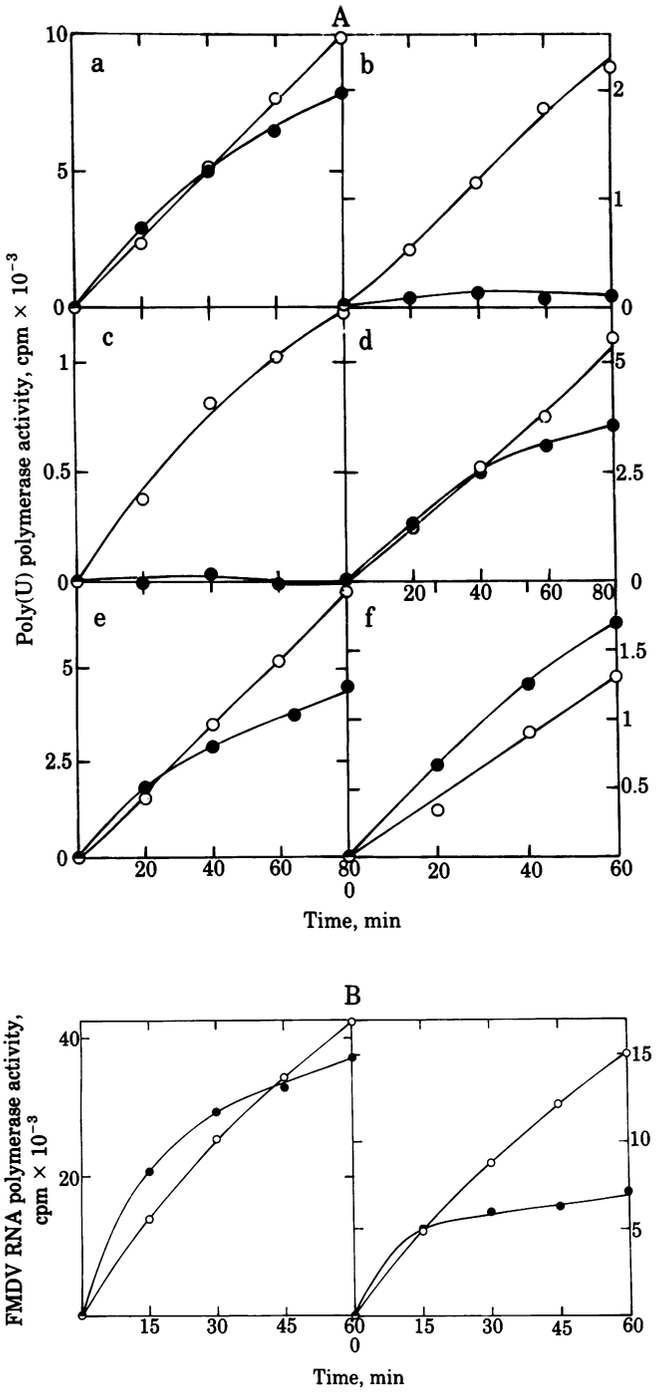

FIG. 5. (*A*) Time course of poly(U) polymerase activity at 33°C and 41°C. The poly(U) polymerase assay mixture (4) (minus added enzyme) was incubated at 33°C or 41°C for 10 min. RNA polymerase isolated by the small-scale purification procedure was brought to room temperature from storage at −20°C immediately before addition to the reaction mixture. Incubation was continued at 33°C (○) or 41°C (●), and 60-μl samples were transferred to 2.3-cm DE-52 DEAE-cellulose discs (Whatman), which were prepared for scintillation counting as described (19). RNA polymerase was purified from the cytoplasm of cells infected with the following viruses: ts^+ (*a*); *ts* 22 (*b*); *ts* 115 (*c*); *ts* 22R (*d*); *ts* 115R (*e*); *ts* 103 (*f*). One pmol of UTP incorporated in the poly(U) polymerase assay is equivalent to 2285 cpm in *a–e* and 3056 cpm in *f*. (*B*) Time course of FMDV RNA-dependent RNA polymerase activity at 33°C (○) and 44.5°C (●). The enzyme from cells infected with ts^+ (*Left*) or *ts* 115 (*Right*) was assayed for RNA-dependent RNA polymerase activity by using the procedure outlined in *A*. One pmol of CTP incorporated in the RNA-dependent RNA polymerase assay is equivalent to 63,220 cpm.

tion of a different base at this site might result in the coding of an amino acid that renders the revertant P56a indistinguishable from the parental protein on IEF gels. Alternatively, a second-site base change in the gene coding for P56a could lead to a charge change in the protein compensating for that induced by the original mutation and resulting in an active polymerase molecule. Resolution of these models awaits nucleic acid sequence analysis for the P56a gene of ts^+ and *ts* 115.

There has been a report that FMDV structural proteins and P56a are phosphorylated (20), although it should be emphasized that the stoichiometry is unknown. Therefore, it could be argued that charge alterations in *ts* 22 and *ts* 115 are due to alterations in the pattern of P56a phosphorylation. By this model, the IEF shifts observed in the P56a of *ts* 22 and *ts* 115 could be due to aberrations in the pattern of phosphorylation brought about by a mutated phosphorylating function. We consider this model to be unlikely because acceptance implies that both "phosphorylated" and "dephosphorylated" forms of P56a are inactive in RNA synthesis. Moreover, we observe no pleiotrophic charge changes in other FMDV-induced polypeptides.

The results presented here provide genetic evidence for the involvement of P56a in FMDV RNA replication. Apart from the obvious usefulness of biochemically defined genetic markers for the 3′ end of the FMDV genome, *ts* RNA polymerase mutants may also find a role in elucidating some aspects of FMDV RNA synthesis both *in vivo* and *in vitro*.

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