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Characterization of Equine Infectious Anemia Virus dUTPase: Growth Properties of a dUTPase-Deficient Mutant

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The putative dUTPase domain was deleted from the polymerase (*pol*) gene of equine infectious anemia virus (EIAV) to produce a recombinant ΔDU*pol Escherichia coli* expression cassette and a ΔDU proviral clone. Expression of the recombinant ΔDU*pol* polyprotein yielded a properly processed and enzymatically active reverse transcriptase, as determined by immunoblot analysis and DNA polymerase activity gels. Transfection of ΔDU provirus into feline (FEA) cells resulted in production of virus that replicated to wild-type levels in both FEA cells and fetal equine kidney cells. In contrast, the ΔDU virus replicated poorly (less than 1% of wild-type levels) in primary equine macrophage cultures, as measured by reverse transcriptase assays. Preparations of ΔDU virus contained negligible dUTPase activity, which confirms that virion-associated dUTPase is encoded in the *pol* gene region between the RNase H domain and integrase, as has been demonstrated previously for feline immunodeficiency virus (J. H. Elder, D. L. Lerner, C. S. Hasselkus-Light, D. J. Fontenot, E. Hunter, P. A. Luciw, R. C. Montelaro, and T. R. Phillips, J. Virol. 66:1791–1794, 1992). Our results suggest that virus-encoded dUTPase is dispensable for virus replication in dividing cells in vitro but may be required for efficient replication of EIAV in nondividing equine macrophages, the natural host cells for this virus.

The lentivirus subfamily of retroviruses includes human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), simian (SIV), feline (FIV), and bovine immunodeficiency viruses, equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus, and visna-maedi virus of sheep (27). While these viruses have some common biological properties, including replication in cells of the immune system, development of persistent infections, and high mutation rates, there is considerable diversity in the virally encoded protein products among the members of the lentivirus subfamily. One example of this diversity can be seen by examining polymerase (*pol*) gene products. In addition to protease (PR), reverse transcriptase (RT), and integrase (IN), EIAV, FIV, caprine arthritis-encephalitis virus, and visna virus contain a fourth *pol* gene product that has been identified as either a pseudoprotease (23) or a dUTPase (24), based on sequence comparisons. This additional protein lies between the RT and IN domains in the *pol* gene (Fig. 1), and Elder et al. (8) have shown that its presence correlates with virion-associated dUTPase activity for EIAV and FIV as well as the type D retroviruses simian retrovirus type 1 and Mason-Pfizer monkey virus (32, 38).

dUTPase, an important enzyme for nucleotide biosynthesis, hydrolyzes dUTP to dUMP and PP_i. The hydrolysis of dUTP provides dUMP as a substrate for thymidylate synthetase and maintains low dUTP-TTP ratios, minimizing uracil incorporation into DNA (2, 11). dUTPase has been isolated from a variety of eukaryotic and prokaryotic organisms, including mammals, plants, *Drosophila melanogaster*, and *Escherichia coli* (4, 10, 14, 16, 30, 46). The ubiquity of dUTPase suggests its importance for DNA replication. For example, dUTPase-deficient *E. coli* cells transiently produce unusually small DNA intermediates during replication and exhibit an elevated frequency of recombination (41). No mammalian cell types mutant in dUTPase have been isolated, which may indicate that dUTPase activity is essential for cell viability.

In addition to certain retroviruses, some herpesviruses and poxviruses have been suggested or proven to encode dUTPase (24, 44, 47), indicating that adequate levels of viral and/or cellular dUTPase may be important for the replication of these viruses. Viral dUTPase is apparently not essential for in vitro replication of herpesviruses (1, 9); however, herpes simplex virus type 1 (HSV-1) dUTPase mutants have recently been shown to exhibit reduced neurovirulence, neuroinvasiveness, and reactivation from latency in vivo (33). dUTPases have been suggested to be both developmentally and cell cycle regulated (7, 13, 15, 28, 30, 39), i.e., their level is low in terminally differentiated and/or nondividing cells. Thus, it is possible that some viruses encode dUTPase activity for the establishment of infections in cell types with low cellular dUTPase activity. We hypothesized that EIAV dUTPase might be advantageous for infection of macrophages, which are proposed to be the primary target cell for this virus (25, 37). We deleted the putative dUTPase of EIAV, expressed the mutant *pol* polyprotein (ΔDU*pol*) in *E. coli*, and assayed for correct polyprotein processing. Subsequently, a dUTPase-deficient (ΔDU) proviral clone was evaluated for replication in established cell lines as well as in cultures of nondividing primary equine macrophages.

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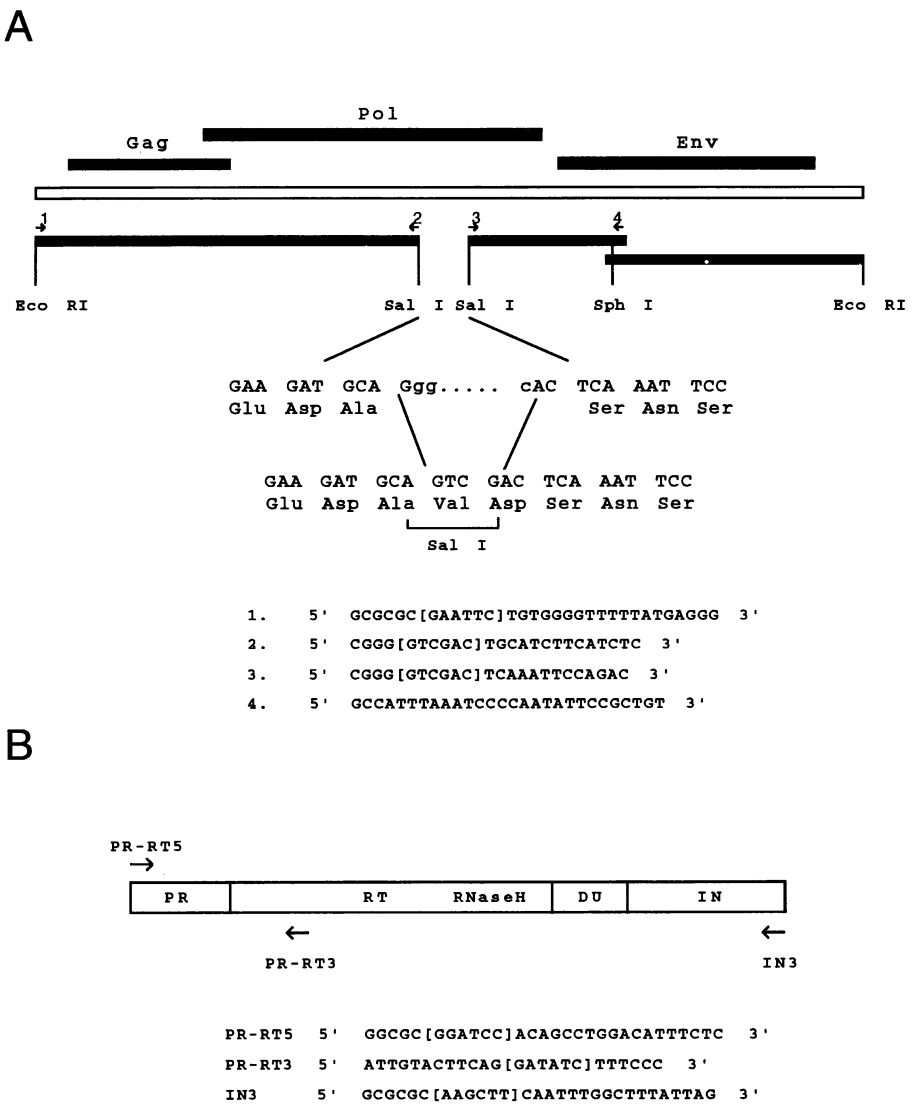

FIG. 1. Construction of an EIAV provirus and a *pol* expression vector deleted in the putative dUTPase region. (A) Construction of ΔDU provirus. Small numbered arrows indicate the locations of PCR primers used to amplify viral fragments for subcloning. The amino acid sequences flanking the deleted region are indicated, as are the two new amino acids produced by the addition of a *Sal*I site to the ΔDU provirus. (B) Construction of EIAV *pol*, ΔDU*pol*, and PR/RT gene cassettes for expression in *E. coli*. The primers used for PCR amplification of the appropriate regions of the *pol* gene are designated by labeled arrows which indicate the direction of the amplification. PCR-amplified fragments were subcloned into an *E. coli* expression vector (20) for further studies.

MATERIALS AND METHODS

Construction of proviral clones and *pol* expression vectors. The dUTPase region of the *pol* gene was deleted from an infectious EIAV provirus recovered from fetal equine kidney (FEK) cell cultures. The provirus, designated pSPEIAV19, was cloned into pLG338-SPORT (6), a derivative of the low-copy-number vector pLG338 (40). As shown in Fig. 1A, the strategy used to create the ΔDU provirus results in an in-frame deletion of 270 bp in the predicted dUTPase coding region and creates a novel *Sal*I site. Polymerase chain reaction (PCR) with primers 1 and 2 (Fig. 1A) and pSPEIAV19 template DNA was used to generate a 3.9-kb fragment containing the 5′ long terminal repeat, the entire *gag* gene, and the sequences encoding PR, RT, and 17 amino acids from the amino terminus of the dUTPase. This fragment was digested with *Eco*RI and *Sal*I and subcloned into *Eco*RI- and *Sal*I-digested pBS+ (Stratagene). The 1.69-kb fragment corresponding to the codons for 27 amino acids from the carboxy terminus of dUTPase, IN, and 197 amino acids from the SU glycoprotein was prepared by PCR with primers 3 and 4 (Fig. 1A). The 1.69-kb PCR fragment was digested with *Sph*I and *Sal*I and ligated to the 3.9-kb fragment in pBS+ to complete the 5′ portion of the ΔDU provirus. A plasmid containing the remainder of the *env* gene was prepared by subcloning a 2.67-kb fragment of pSPEIAV19 extending from the *Sph*I site in the *env* gene to the end of the 3′ long terminal repeat into pLG338-SPORT.

In initial experiments, the 5′ and 3′ clones were digested with *Sph*I and ligated overnight at 16°C prior to transfection. As a control for ligation and transfection, the 5′ portion of

the infectious proviral clone pER (31) was digested with *Sph*I and ligated to the 3′ portion of pSPEIAV19. For later experiments, a full-length ΔDU proviral clone was assembled in pLG338-SPORT.

The construction of EIAV *pol*, ΔDU*pol*, and PR/RT gene cassettes used for expression in *E. coli* is illustrated in Fig. 1B. Primer pair PR-RT5 and PR-RT3 (Fig. 1B) amplify a fragment corresponding to the entire PR region and a portion of RT. The PR-RT5 oligonucleotide contains a *Bam*HI site at the 5′ end for cloning purposes; the PR-RT3 oligonucleotide overlaps a unique *Eco*RV site in RT. This PCR fragment was digested with *Bam*HI and *Eco*RV and subcloned into a previously described expression vector (p6HEIAVRT) containing the EIAV RT gene (21) to generate an expression vector containing the entire PR/RT coding region. The PR-RT5 and IN3 primer pair (Fig. 1B) generate either EIAV *pol* or ΔDU*pol* when used with the appropriate templates (pSPEIAV19 or ΔDU 5′ in pBS+, respectively). The *pol* gene products were expressed with amino-terminal extensions of six histidines, as described previously for HIV RT (20).

Immunoblots of bacterial lysates. *E. coli* cells containing expression vectors for EIAV *pol* (p6HEIAVpol), ΔDU*pol* (p6HEIAVΔDUpol), or PR/RT (p6HEIAVPRRT) were induced with 200 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml for 1 h. As described previously (20), bacteria were pelleted, lysed in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (19) containing 0.72M β-mercaptoethanol, and fractionated by electrophoresis on denaturing 10% polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose filters, which were blocked overnight with 3% bovine serum albumin and incubated for 1 h with mouse antiserum raised against purified recombinant EIAV RT (21). Goat anti-mouse immunoglobulin-horseradish peroxidase conjugate (Pierce) was used as the second antibody, and color reaction was developed with a CN/DAB substrate kit (Pierce).

DNA polymerase activity gels. Bacterial pellets from cultures induced with IPTG were lysed in 50 mM Tris-HCl (pH 7.2)–15% sucrose–0.8 mg of lysozyme per ml. Lysates were mixed with equal quantities of 1× activity gel sample buffer (50 mM Tris-HCl [pH 8.0], 5% glycerol, 2 mM EDTA, 1% SDS, 0.02% bromophenol blue, 1 mg of bovine serum albumin per ml, 5 mM dithiothreitol) and stored at −20°C until just prior to gel loading. Samples were heated at 37°C for 5 min and loaded immediately onto 10% polyacrylamide gels containing DNase-treated salmon sperm DNA (50 μg/ml) and 2 mM EDTA. Gels were then treated as described previously for HIV RT (3) except that [α-^{32}P]dCTP was used instead of [α-^{32}P]TTP. Briefly, gels were soaked at room temperature for 24 to 36 h in several changes of a solution of 50 mM Tris-HCl (pH 8.0)–1 mM EDTA and then incubated in a solution of 25 mM Tris-HCl (pH 8.0)–8 mM $MgCl_2$–40 mM KCl for 12 h at room temperature. Following this incubation, 50 μM each dATP, TTP, and dGTP and 1 μCi of [α-^{32}P]dCTP per ml were added, and the gels were incubated overnight at 37°C with shaking. The reactions were stopped by washing with at least three changes of a solution of 5% trichloroacetic acid–1% sodium PP_i at 4°C for at least 24 h. The gels were dried, and autoradiography was performed with Kodak X-Omat AR film at room temperature for 4 to 8 h.

Cells and viruses. A feline cell line (FEA) and primary FEK cells were maintained in Eagle's minimal essential medium with Earle's salts, supplemented with 5% fetal bovine serum (BioWhittaker), 25 U of penicillin, 25 μg of streptomycin sulfate, and 0.5 μg of amphotericin B (Fungizone; GIBCO/BRL) per ml. Equine macrophages were prepared from whole blood by centrifugation through Ficoll-Hypaque and maintained in RPMI 1640 plus 10% fresh autologous horse serum (42).

Virus stocks were prepared by transfection of molecular clones into FEA cells. Clarified supernatants from transfected FEA cell cultures were used as stocks for infection of FEA cells, FEK cells, and equine macrophages.

Transfections. FEA cells were plated to 20% confluence in 60-mm petri dishes and transfected with proviral DNA by calcium phosphate precipitation with the GIBCO/BRL Transfinity kit. After exposure to DNA for 3 h, the cells were washed in phosphate-buffered saline, pH 7.4, and subjected to glycerol shock (36). The cultures were grown to confluence and then reseeded into 25-cm^2 flasks for the duration of the experiments.

RT assays. Virus growth was measured by determination of RT activity in culture supernatants. RT activity was determined for FEA and FEK cell supernatants essentially as described by Willey et al. (43). Reaction mixes contained 0.25 optical density unit of poly(rA) · poly(dT)$_{12-18}$, 50 mM Tris-HCl (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, and 0.5 μCi of [^{32}P]TTP (3,000 Ci/mmol). Fifty microliters of reaction cocktail was added to 10 μl of culture supernatant (clarified and containing 1% Triton X-100) and incubated at 37°C for 45 min. Reactions were terminated by spotting 5 μl of the reaction mix onto DE81 filter paper and washing twice for 5 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and twice for 5 min each in 95% ethanol. The filter papers were dried, and TTP incorporation was determined by Cerenkov counting (18).

RT activity in equine macrophage culture supernatants was determined by the method of Gregerson et al. (12) with [^{3}H]TTP rather that [^{32}P]TTP. Macrophages were maintained in 12-well plates, and infections of approximately 10^5 cells, with either pSPEIAV19 or ΔDU virus, were performed in duplicate. At each time point assayed, virus was pelleted (1 h, 15,000 × *g*) from 1 ml of culture supernatant, and the virus pellet was resuspended in 10 μl of buffer containing 50 mM Tris (pH 8.3), 20 mM dithiothreitol, and 0.25% Triton X-100.

dUTPase assays. Virus-containing culture supernatants were underlayered with 20% glycerol and centrifuged for 2 h at 25,000 rpm in an SW41 rotor. The resulting pellet was resuspended in 50 mM Tris-HCl (pH 8.0) and stored at −20°C. Protein concentrations were determined with the Bio-Rad protein assay reagent, and equal amounts of protein from wild-type and ΔDU viruses were assayed for dUTPase activity. dUTPase activity in the crude virus preparations was measured as described by Williams (44). Reaction mixtures contained, in a total volume of 100 μl, 50 mM Tris-HCl (pH 8.0), 2 mM β-mercaptoethanol, 1 mM $MgCl_2$, 0.1% (wt/vol) bovine serum albumin, 2 mM *p*-nitrophenylphosphate, a total of 0.1 mM dUTP ([^{3}H]dUTP at 50 μCi/μmol), and 3 μg of protein. The reaction mixtures were incubated at 37°C for 20 min, and reactions were terminated by spotting 20 μl onto DE81 filter paper and immediate washing in a solution of 4 M formic acid–1 mM ammonium formate. Filters were further processed as described previously (46). To verify that the enzymatic activity being measured was specific for dUTP, dCTP and TTP were substituted for dUTP in the assay mixture. The same nucleotide concentrations were used, i.e., 0.1 mM dCTP ([^{3}H dCTP at 50 μCi/μmol) or 0.1 mM TTP ([*methyl*-^{3}H]TTP at 50

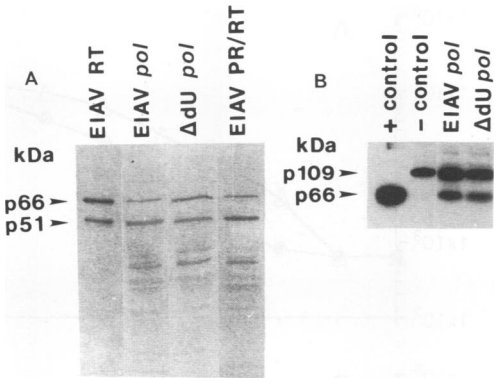

FIG. 2. Analyses of EIAV *pol* and ΔDU*pol* polyprotein processing in *E. coli*. (A) Immunoblot analysis of lysates from *E. coli* expressing the EIAV *pol* gene, ΔDU*pol*, or PR/RT. The control for correct processing of RT is purified recombinant EIAV RT, which shows two immunoreactive bands corresponding to 66- and 51-kDa polypeptides, as seen previously (21). (B) In situ DNA polymerase assays of lysates from cultures expressing EIAV *pol* or ΔDU*pol*. The cultures were induced for 45 min prior to lysis. The negative-control culture contains the EIAV S3 (putative *rev*) open reading frame in the same expression vector. The positive control is purified, recombinant HIV-1 RT, showing the DNA polymerase activity associated with the 66-kDa polypeptide. The 109-kDa DNA polymerase observed in all of the lysates corresponds to *E. coli* DNA polymerase I.

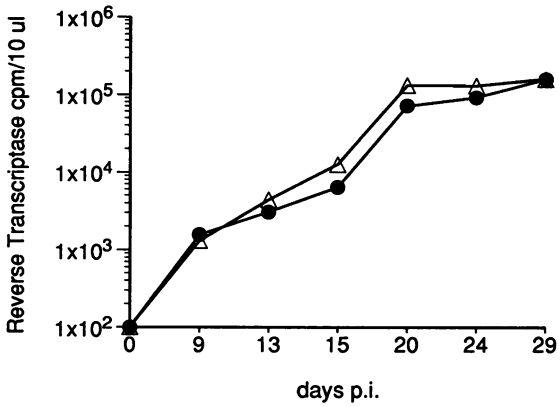

FIG. 3. Replication kinetics of ΔDU (●) and wild-type (△) proviruses after transfection into uninfected FEA cells. RT activity was monitored as an indication of virus replication. p.i., postinfection.

μCi/μmol). [^{3}H]dUTP (21 Ci/mmol) was purchased from Amersham, and [^{3}H]dCTP (31 Ci/mmol) and [^{3}H]TTP (13 Ci/mmol) were purchased from ICN Biochemicals.

PCR evaluation of virus stocks. The purity of the ΔDU virus stock was determined by PCR amplification of a portion of the *pol* gene. Cells infected with ΔDU or wild-type virus were collected by trypsinization. Cells were lysed in a buffer containing 50 mM KCl, 15 mM $MgCl_2$, 10 mM Tris (pH 8.5), 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 0.5 mg of proteinase K per ml at 55°C for 1 h (26). Thirty cycles of PCR were performed (1 min at 94°C for denaturation, 2 min at 50°C for annealing, and 2 min at 72°C for extension) with *pol*-specific primers dU1 (5′-TTGGCT CCCAGAAATAGTATATACA-3′) and dU2 (5′-GCATGT ATGTATCCTGAATTTGAC-3′). The PCR products were analyzed by agarose gel electrophoresis and Southern hybridization.

RESULTS

To assess the requirement for the dUTPase coding domain for the expression and replication of EIAV, we constructed an expression cassette (ΔDU*pol*) and a provirus (ΔDU) in which the dUTPase-coding region was deleted. We initially characterized the expression and processing of the viral RT from the ΔDU*pol* expression cassette to ensure that we had not grossly disrupted protein expression and processing. Subsequently, the ΔDU provirus was used to transfect FEA cells, and the resulting virus stock was characterized to ensure purity and the absence of dUTPase activity. Finally, replication of the ΔDU virus stock in equine cells was compared with that of the parental wild-type virus.

***pol* polyprotein expression and processing.** Immunoblot analysis with mouse serum raised against purified recombinant EIAV RT (21) was performed on EIAV PR/RT, *pol*, and ΔDU*pol* gene constructs expressed in *E. coli*. As shown in Fig. 2A, the blots revealed that the ΔDU*pol* construct yielded apparently correctly processed RT composed of polypeptides of 66 and 51 kDa. The smaller immunoreactive band observed for all three constructs is most likely an RT breakdown product. Figure 2B shows significant DNA-dependent DNA polymerase activity associated with the 66-kDa RT subunit for both the *pol* and ΔDU*pol* constructs. This activity comigrates with the 66-kDa polymerase activity of purified recombinant HIV RT, used as a positive control. A negative control shows only the DNA polymerase activity associated with the 109-kDa polypeptide of *E. coli* DNA polymerase I. Thus, the in-frame deletion of the dUTPase-coding region does not inhibit either expression or proteolytic processing of or the DNA polymerase activity associated with EIAV RT.

Western blots of the *pol* and ΔDU*pol* constructs were also performed with horse immune serum. These blots revealed an additional immunoreactive band of 32 kDa, which is most likely the IN polypeptide (data not shown). We attempted to verify the expression and processing of active IN protein from the ΔDU proviral construct by performing Southern blots with DNA extracted from both ΔDU- and wild-type-infected FEA cells. In both cases, approximately 50% of the proviral DNA migrated with the high-molecular-weight DNA fraction and 50% migrated as unintegrated linear molecules (data not shown). The observed ratio of hybridization signal associated with the high-molecular-weight fraction to free unintegrated molecules is similar to that reported previously for EIAV (34) and strongly suggests that the *pol* polyprotein is processed to produce a functional IN protein.

Production and characterization of ΔDU virus stocks. To produce ΔDU virus stocks, FEA cells were transfected with wild-type and ΔDU proviral clones as described in Materials and Methods. As shown in Fig. 3, RT activity was detected in the viral supernatants at approximately 2 weeks posttransfection and rose quickly thereafter, indicating the production of infectious virions capable of spreading throughout the culture. In multiple transfections, we observed no consistent difference in virus yields between the ΔDU and wild-type proviruses in FEA cells, as measured by free RT activity in cells.

To verify the purity of the ΔDU virus stock, the supernatant from transfected FEA cells was passed onto fresh

TABLE 1. Viral dUTPase activity

Virus	dUTPase activity[a] (% conversion)		
	dUTP	TTP	dCTP
Wild type	95.5	18.5	0
ΔDU	16.9	14.6	0

[a] Values represent percent conversion of the indicated nucleoside triphosphate to nucleoside monophosphate plus PP_i and are the averages of three experiments.

uninfected FEA cells. RT levels were monitored to verify virus replication, and cells were collected, lysed, and subjected to PCR analysis with *pol*-specific primers. The PCR products were analyzed by ethidium bromide staining and Southern hybridization. We observed no PCR products representative of the parental wild-type virus in the ΔDU virus-infected cells (data not shown).

Finally, since the putative dUTPase region of EIAV was only partially deleted, we wished to verify that ΔDU virus preparations lacked the activity associated with wild-type virus. As shown in Table 1, conversion of dUTP to dUMP and PP_i by wild-type virus was 95% after 20 min at 37°C. In contrast, conversion of dUTP to dUMP and PP_i by the ΔDU virus was no greater than the background levels observed for TTP. The low level of hydrolysis of TTP (15 to 18%) and dCTP (not measurable) indicates that the assay is specifically measuring dUTPase activity. Thus, the *pol* deletion does encompass the region responsible for virion-associated dUTPase activity. The truncated polypeptide encoded by the mutant virus has no measurable dUTPase activity.

Replication of ΔDU virus in equine cells. To assess the ability of the ΔDU virus to replicate in equine cells, equivalent amounts (based on RT activity) of the ΔDU and wild-type viruses were used to infect FEK cells and primary equine macrophages. As shown in Fig. 4, there is no significant difference in the replicative ability of the ΔDU and wild-type viruses in FEK cells. At the lowest dose of input virus, we observed that 50% less ΔDU virus than wild-type virus was produced; however, there was still more than 10^5 cpm of RT activity per 10 μl in the cell supernatant.

In contrast, as shown in Fig. 5, the ΔDU virus is significantly impaired in its ability to replicate in macrophages. Equine peripheral blood cell macrophage cultures were infected with either wild-type or ΔDU virus stocks at 200,000 cpm (2×10^4 50% tissue culture-infective doses [$TCID_{50}$] of infectious virus) or 20,000 cpm (2×10^3 $TCID_{50}$ of infectious virus). An infectivity titration on FEA cells revealed that there was no significant difference in the specific infectivity (cpm per $TCID_{50}$) of the virus stocks. At equivalent doses of input virus, the ΔDU virus stock yielded (based on RT activity) less than 5% of the virus obtained from the wild-type-infected cultures. In order to determine whether the RT activity seen in the ΔDU-infected cultures represented infectious particles, we performed infectivity titrations on day 9 postinfection. The high-dose wild-type-infected cells yielded an average of 5.6×10^3 $TCID_{50}$/ml, whereas the high-dose ΔDU-infected cells yielded an average of 2.8×10^1 $TCID_{50}$/ml. The low-dose wild-type- and ΔDU-infected cells yielded 2.8×10^4 and $<5 \times 10^0$ $TCID_{50}$/ml, respectively, at 9 days postinfection. The lower infectivity titer of the high-dose wild-type-infected cells than of the low-dose wild-type-infected cells at 9 days postinfection presumably reflects the fact that the high-dose wild-type-infected cells

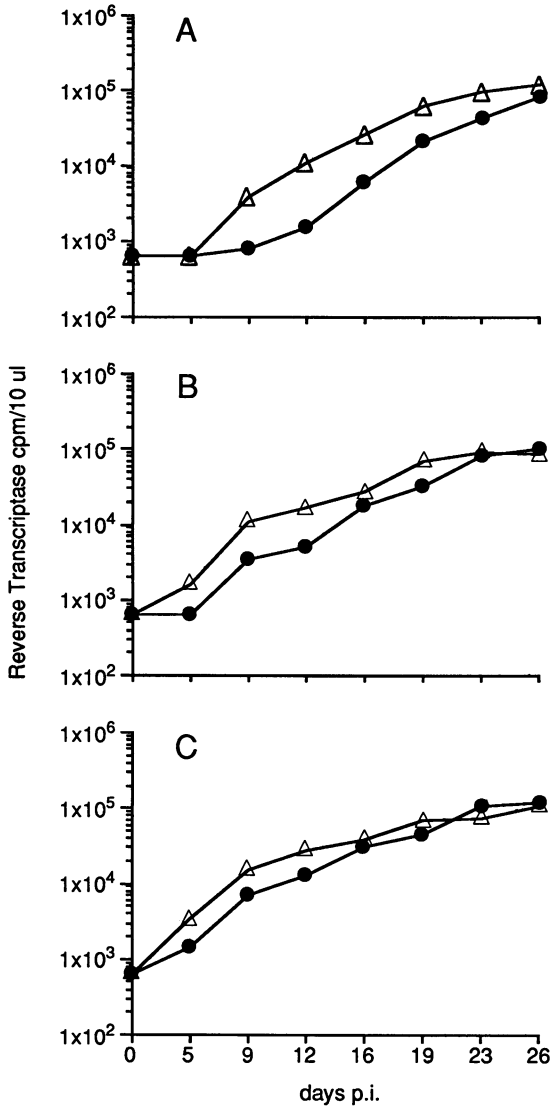

FIG. 4. Growth of ΔDU (●) and wild-type (△) EIAV in FEK cells. Equivalent amounts (based on RT units) of cell-free ΔDU and wild-type viruses were used to infect FEK cell cultures. Three doses of input virus were used in order to determine whether the replication phenotype of the ΔDU virus is influenced by the initial multiplicity of infection: (A) 10^4 cpm; (B) 10^5 cpm; and (C) 10^6 cpm. Virus replication was monitored by RT assays as described in the text. p.i., postinfection.

exhibited extensive cytopathic effects by 7 days postinfection, versus 9 days for the low-dose wild-type-infected cells.

We were unable to detect any cytopathic effects for the ΔDU-infected cells at either virus dose. Figure 6 shows Geimsa-stained macrophage cultures at 9 days postinfection. The monolayer has been almost completely destroyed in the wild-type-infected culture, while there are no obvious cytopathic effects in the ΔDU-infected culture compared with the mock-infected control.

DISCUSSION

Compared with the primate lentiviruses, EIAV and several other nonprimate lentiviruses have an additional coding

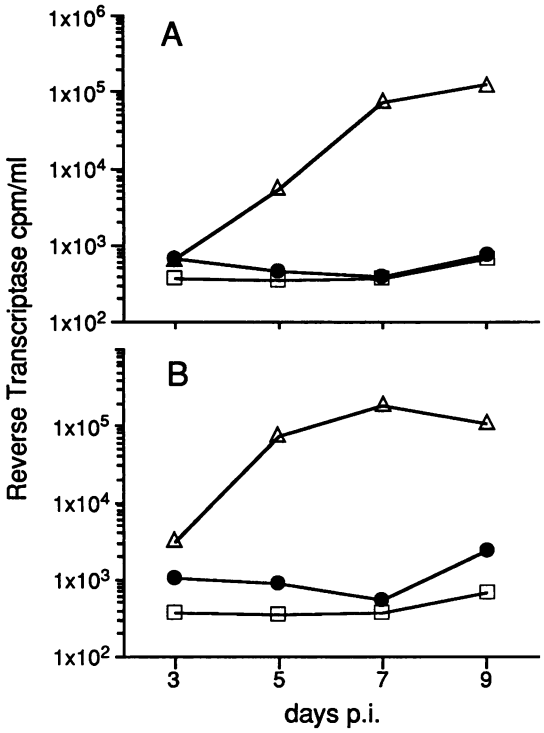

FIG. 5. Equivalent amounts of cell-free ΔDU (●) and wild-type (△) viruses were used to infect primary equine macrophage cultures. Two doses of input virus were used to determine whether the replication phenotype of the ΔDU virus is influenced by the initial multiplicity of infection. (A) Infection with 2×10^5 cpm (2×10^4 $TCID_{50}$) of input virus. The results shown are the averages for duplicate cultures. (B) Infection with 2×10^4 cpm (2×10^3 $TCID_{50}$) of input virus. The results shown are the averages for triplicate cultures. □, Mock-infected cultures. The $TCID_{50}$ determinations for the virus stocks used in this experiments were done on permissive (FEA) cells. p.i., postinfection.

region in the *pol* gene. This additional protein has been suggested to be either a pseudoprotease or a dUTPase, based on sequence comparisons (23, 24). Recently, this region of the FIV *pol* gene was shown to encode a polypeptide with dUTPase activity (8). The same authors also showed that this activity was associated with gradient-purified preparations of EIAV. Since dUTPase activity was not detected with viral preparations of retroviruses lacking this conserved region, we assumed that the region between RT and IN in the EIAV *pol* gene did encode dUTPase.

To test whether the putative dUTPase of EIAV was important for viral replication, we deleted a large portion of the dUTPase region of the *pol* gene. The deletion corresponds to conserved motifs 1 to 4 of dUTPase, as described by McGeoch (24). Interestingly, the crystal structure of the trimeric *E. coli* dUTPase has recently been solved, and certain residues from motifs 1 to 4 are suggested to be critical for activity, while motif 5 appears to perform a subunit contact function (4). By analogy, we assumed that deletion of motifs 1 to 4 would render EIAV dUTPase inactive. Indeed, as Table 1 indicates, the activity associated with wild-type EIAV is absent in the ΔDU mutant. To verify that the deletion did not interfere with correct polyprotein processing, the Δ*DUpol* gene was cloned into a bacterial expression vector and its processing was compared with that of the intact *pol* polyprotein (Fig. 2). In addition, IN function was monitored indirectly by Southern blots of ΔDU-infected FEA cells, and we observed no difference in the amount of proviral DNA in the high-molecular-weight fraction between wild-type- and ΔDU-infected cultures. Although we have not analyzed proviral integration in ΔDU-infected macrophages, we believe that it is unlikely that IN would be functional in FEA cells but nonfunctional in macrophages. Thus, the deleted polyprotein appears to be processed accurately, indicating that (i) the dUTPase does not perform an essential function in *pol* polyprotein processing and (ii) the inability of ΔDU virus to replicate to high titer in macrophages is unlikely to result from failure to express and process *pol* proteins. We are currently addressing all steps in the viral life cycle to determine the specific block(s) to ΔDU replication in macrophages.

As indicated in Fig. 3 and 4, the ΔDU virus is able to replicate well in FEA and FEK cells. RT levels for the wild-type and mutant viruses rise at approximately the same time after transfection or infection, and the mutant virus replicates to high levels. PCR analysis of cells infected with ΔDU virus verified that these cultures were not contaminated with wild-type virus. The fact that ΔDU EIAV replicates well in certain cell lines correlates with findings seen with dUTPase-deficient HSV-1. Studies with HSV-1 have shown that loss of viral dUTPase activity can apparently be compensated for by host cell dUTPase activity in vitro (1, 9). Indeed, some evidence suggests that intact herpesviruses (i.e., those with viral dUTPase) actually downregulate the cellular dUTPase activity by a phosphorylation mechanism (22, 45).

Because neither FEA nor FEK cells are true representatives of the cell type in which EIAV replicates in vivo, equine macrophage cultures were infected with ΔDU and wild-type viruses. In this case, only the control virus was able to replicate to high levels and cause significant cytopathic effects (Fig. 5 and 6). One explanation for the limited replication of the ΔDU virus in macrophages is that cellular dUTPase levels would be expected to be lower in these nondividing cells than in actively growing FEK and FEA cells. Evidence from several laboratories indicates that dUTPase levels are regulated both in the context of the cell cycle and in differentiation programs. Different levels of dUTPase activity throughout the cell cycle have been demonstrated for temperature-sensitive Chinese hamster cell lines, i.e., minimal at G_0 and maximal at S phase (7). In rats given partial hepatectomy or treated with phenylhydrazine to induce anemia, increased dUTPase levels correlated with liver cell growth and the multiplication of erythroid cells in the spleen (13, 15). In the higher plant *Allium cepa*, definitive evidence exists for an increase in dUTPase activity at the G_1/S phase boundary, with high levels throughout the S phase, declining to very low levels in G_2 that remain low at the beginning of G_1. Additionally, terminally differentiated cells which no longer divide mitotically showed reduced levels of dUTPase activity (30). In *D. melanogaster*, a specific inhibitor of dUTPase which tightly restricts the expression of the enzyme to only certain embryonic stages (i.e., prior to differentiation of tissues) has been identified (28). In adult rabbits, tissues which are terminally differentiated and/or not dividing mitotically (i.e., brain and liver) also show reduced dUTPase levels (39), in contrast to the same tissues in neonatal rabbits.

Of relevance to these findings, HSV infects nervous tissue (35), while lentiviruses infect T lymphocytes and/or cells of the monocyte/macrophage lineage (27). These cell popula-

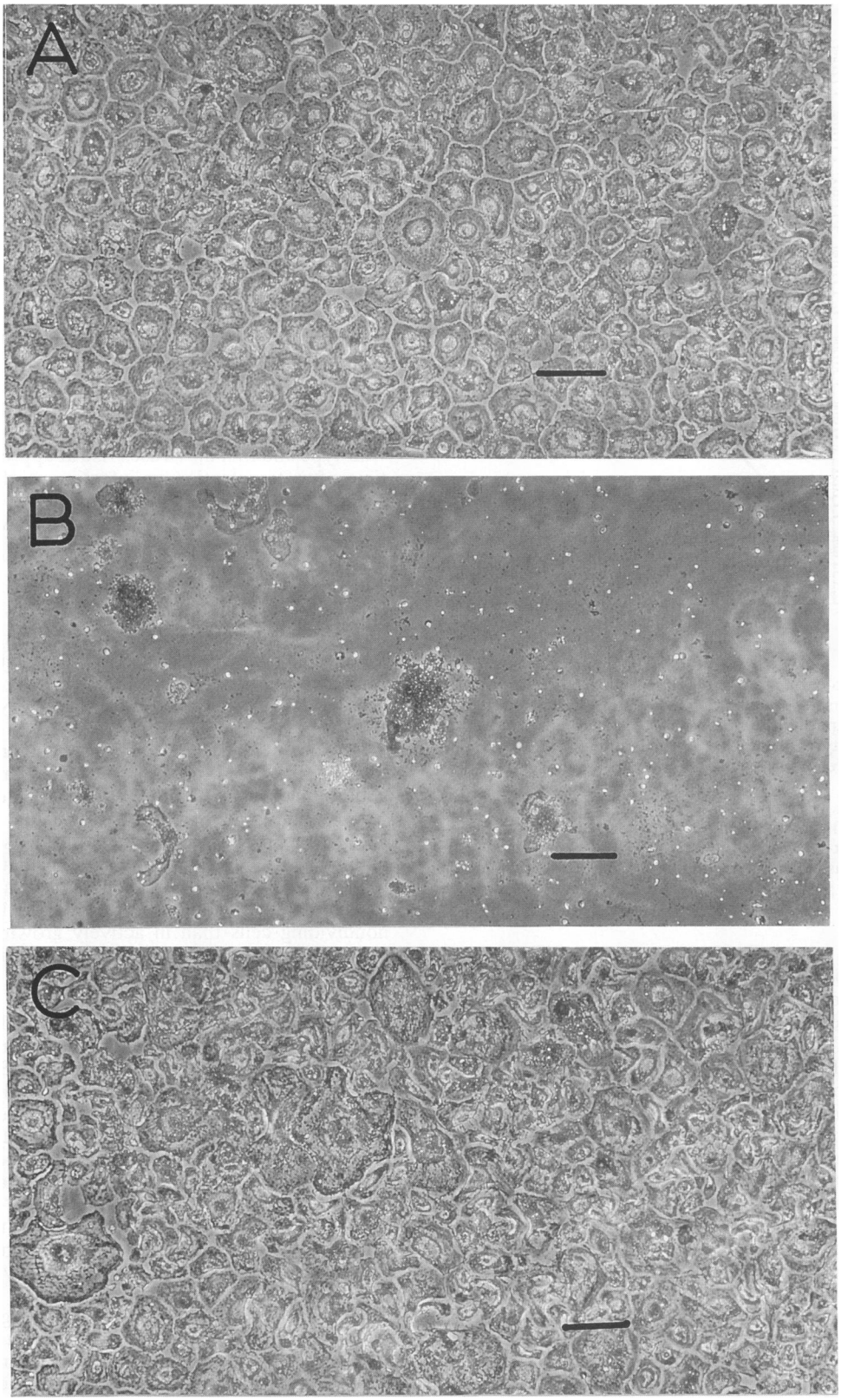

FIG. 6. Comparison of the cytopathic effects of wild-type and ΔDU proviruses in equine macrophage cultures. Equine adherent peripheral blood cell cultures in 12-well dishes (each well containing about 10^5 cells) were infected with 200,000 cpm (2×10^4 $TCID_{50}$ units) of wild-type or ΔDU virus. Virus was allowed to adhere for 1 h at 37°C, the inoculum was removed, and 2 ml of RPMI 1640 containing 10% fresh autologous horse serum was added. RT activity in the supernatant fluid was monitored, and the appearance of the cultures is shown at 9 days postinfection after fixation with 1% paraformaldehyde in phosphate-buffered saline and staining with Giemsa stain. (A) Mock-infected cells; (B) wild-type-infected cells; (C) ΔDU-infected cells. Bars, 100 μm.

tions may have low dUTPase levels, and thus a virally encoded enzyme could assist in establishment of infections in these cell types. In fact, recent studies have demonstrated the importance of HSV-1 dUTPase in pathogenesis (33). Ideally, we would like to be able to show directly that the presence of EIAV dUTPase is responsible for the growth advantage of wild-type virus in macrophages. This could be done by transfecting macrophages with a plasmid capable of expressing EIAV dUTPase prior to infection with ΔDU virus. Unfortunately, such complementation experiments are technically difficult for several reasons. One is the difficulty of adequate DNA transfer into primary macrophages, and a second is the limited viability of these cells in culture. We are currently attempting to develop a model system in which serum starvation of FEA cells is used to reduce cellular dUTPase levels. This system may be more amenable to complementation experiments. In addition, we are trying to quantitate macrophage dUTPase levels.

In contrast to the studies presented here, which indicate that EIAV dUTPase apparently plays an important role in macrophage infection, the primate lentiviruses HIV and SIV, while lacking the equivalent domain, are capable of replication in nondividing cells of the macrophage lineage (5). Many strains of HIV are noncytopathic in macrophages, and the virus titers produced are lower that those produced in activated T lymphocytes. Thus, it is possible that in the case of EIAV, dUTPase is required in vivo for the production of high levels of infectious virus during febrile episodes (when the titer of virus can reach 10^6 horse-infectious doses per ml in whole blood and serum [17]).

The specific function of dUTPase in the retrovirus life cycle is unclear at this time; however, conversion of dUTP to dUMP plus PP_i could supply dUMP as a substrate for thymidylate synthetase, increasing cellular TTP pools. In this regard, some preliminary evidence concerning reverse transcription of HIV genomes in macrophages (29) showed that the addition of nucleotide precursors to macrophage cultures altered the kinetics of reverse transcription. In particular, completion of reverse transcription was accelerated in nucleotide-treated cultures. Additionally, a retroviral dUTPase may result in reduced mutation levels, as suggested previously (8). During reverse transcription, it could function to reduce local dUTP levels, reducing dUTP misincorporation during reverse transcription. In the case of EIAV-infected macrophages, high cellular dUTP levels could contribute to high mutation rates due to hyperrecombination (as is observed in dUTPase-deficient *E. coli*) or an accumulation of point mutations. Either event could lead to a loss of infectious virus. Further studies with dUTPase-deficient lentiviruses should reveal the importance of dUTPases in retroviral pathogenesis.

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