Replication In Vitro and In Vivo of an Equine Infectious Anemia Virus Mutant Deficient in dUTPase Activity

DREW L. LICHTENSTEIN,¹ KEITH E. RUSHLOW,^{1†} R. FRANK COOK,² MICHELLE L. RAABE,¹
CHRISTINE J. SWARDSON,^{3‡} GARY J. KOCIBA,³ CHARLES J. ISSEL,²
AND RONALD C. MONTELARO^{1*}

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261¹; Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546²; and Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210³

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As an important enzyme in DNA synthesis, dUTPase is present in a wide variety of organisms and viruses and has been identified as a component of the equine infectious anemia virus (EIAV) *pol* gene. The role of EIAV dUTPase, designated DU, in virus replication in vitro and in vivo was investigated with a recently described infectious molecular clone of EIAV. A deletion mutant that was deficient in dUTPase activity was constructed, and its replication kinetics was examined in fetal equine kidney (FEK) cells and primary equine bone marrow macrophage (EBMM) cells. In FEK cells, which are permissive for EIAV replication, the mutant virus replicated as well as the parental virus. In primary cultures of EBMM cells, which are primary targets of EIAV infection in vivo, the DU mutant showed delayed replication kinetics and replicated to a lower extent than did the parental virus. As the multiplicity of infection decreased, the difference between the parental and mutant viruses increased, such that at the lowest multiplicity of infection tested, there was over a 100-fold difference in virus production. The mutant virus was also much less cytopathic. The role of DU in replication in vivo was examined using a Shetland pony model of EIAV infection. Shetland ponies that were infected with the parental and mutant viruses showed transient virus RNA levels in plasma approximately 5 to 10 days postinfection. The peak virus levels in plasma (as measured by a quantitative reverse transcriptase PCR assay) were 10- to 100-fold lower in the mutant virus-infected animals than in the animals infected with the parental virus. However, ponies infected with the mutant virus mounted similar antibody responses despite the marked differences in virus replication. These studies demonstrate that EIAV DU is important for the efficient replication of the virus in macrophages in vitro and in vivo and suggests that variations in the DU sequence could markedly affect the biological and pathogenic properties of EIAV.

The relative genetic simplicity of the equine infectious anemia virus (EIAV) genome and the availability of an animal model for investigating EIAV pathogenesis provide a unique model system in which to examine the contribution of specific viral genes in lentivirus replication, persistence, and pathogenesis. EIAV, a member of the lentivirus subfamily of retroviruses, encodes the three basic retrovirus gene products, Gag, Pol, and Env. Only three auxiliary gene products, Tat (the viral transactivator protein), Rev (the regulator of virus splicing), and S2 (a protein of unknown function), have been identified to date (8, 31, 34, 35). It was recently reported that a subset of retroviruses, including EIAV and feline immunodeficiency virus (FIV), contain a virion-associated dUTPase (EC 3.6.1.23) activity (9). dUTPase catalyzes the conversion of dUTP to dUMP and PP_i. The gene encoding dUTPase has been mapped within the *pol* gene for EIAV and FIV and within the *gag* gene for mouse mammary tumor virus (27, 54, 58). The lentivirus dUTPase gene has been designated DU (9).

In addition to EIAV and FIV, other nonprimate lentiviruses that encode a DU gene include visna virus and caprine arthritis

encephalitis virus. Other retroviruses that encode a dUTPase activity include Mason-Pfizer monkey virus and mouse mammary tumor virus (2, 9). Viruses other than retroviruses that encode a dUTPase gene include the poxviruses vaccinia virus and Orf virus and the herpesviruses herpes simplex virus (HSV) types 1 and 2, equine and bovine herpesviruses, and cytomegalovirus (3, 9, 27, 33). The enzyme has also been described in a wide variety of organisms such as mammals, plants, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Escherichia coli* (7, 15, 18, 22, 37, 42, 60).

The presence of dUTPase in a wide variety of organisms, as well as in several virus families, suggests the enzyme provides a critical function in DNA replication. In addition to supplying dUMP, a key substrate in the de novo synthesis of dTTP, dUTPase maintains a low dUTP/dTTP ratio such that uracil, which is not normally a component of DNA in most systems, is excluded from newly synthesized DNA (28). Furthermore, dUTPase has been shown to be an important neurovirulence factor for HSV-1 (43). The contribution of lentivirus DU to replication has also been examined. Studies with mutants of the EIAV and FIV DU show that the enzyme is not required for replication in fetal equine kidney (FEK) cells and Crandell feline kidney cells, respectively (54, 58). However, in both cases, efficient virus replication in cells of the monocyte/macrophage lineage occurred only in the presence of a functional DU gene (54, 58). An important caveat of these observations is that the replication phenotype exhibited in vitro is not always a good predictor of the phenotype displayed in vivo. This is

* Corresponding author. Mailing address: Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. Phone: (412) 648-8869. Fax: (412) 624-1401.

† Present address: Paravax Inc., Fort Collins, CO 80525.

‡ Present address: Department of Pathology, Colorado State University, Fort Collins, CO 80523.

clearly shown by studies demonstrating that mutants of simian and human immunodeficiency viruses (SIV and HIV, respectively) deficient in Nef protein synthesis behave differently in cell culture and in animal infection models (11, 23–25, 47, 53). In this report, we compare the replication properties of an infectious EIAV mutant deficient in dUTPase activity in both in vitro and in vivo models. The in vitro replication kinetics were compared in two types of cells, one routinely used for cell culture production of EIAV and the other a target of infection in vivo. In addition, the replication properties of and host immunological responses to the mutant virus were studied in vivo using a Shetland pony model of EIAV infection.

MATERIALS AND METHODS

Cells. FEK cells were grown at 37°C in minimum essential medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, Ga.), penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM), and nonessential amino acids (GibcoBRL). Primary equine bone marrow macrophage (EBMM) cells were prepared as described previously (51). Briefly, mononuclear cells were isolated from equine bone marrow and prepared by centrifugation through Histopaque (Sigma, St. Louis, Mo.). Following a wash in Hanks balanced salt solution (GibcoBRL), cells were seeded in 75-cm² flasks (Corning, Corning, N.Y.) in minimum essential medium alpha medium (GibcoBRL) supplemented with 10% heat-inactivated horse serum (Sigma), penicillin G (100 U/ml), streptomycin (100 µg/ml), and glutamine (2 mM). Nonadherent cells were removed after an overnight incubation at 37°C, and any remaining adherent cells were incubated for an additional 24 to 48 h. The adherent cells were detached with 2.6 mM EDTA in Hanks balanced salt solution and seeded into 24-well plates (Corning) at 10⁵ cells per well.

Viruses. The construction and characterization of PV 19-2, an infectious molecular clone of the pathogenic strain of EIAV, have been described previously (39). Standard molecular cloning techniques (30) were used to construct the proviral clone PV 19-2-6A, which differed from PV 19-2 only within the long terminal repeat U3 region. A 165-bp fragment from the *Mlu*I site to the 3' end of the viral genome from PV 19-2 was replaced with the corresponding long terminal repeat U3 region from clone P3.2.5 (38). Thus, proviral clone PV 19-2-6A contains two copies of the putative transcription regulatory element CAAT. A deletion in the DU coding sequence was generated by removing a 330-bp *Sst*I restriction fragment from a *Kpn*I-*Pst*I *pol* subclone of PV 19-2-6A. This deleted segment was then subcloned back into a full-length provirus backbone as an *Sst*I-*Nco*I fragment to create the mutant provirus clone PV 19-2-6A-ΔDU. Clones were subsequently screened for the additional *Hind*III site created by the ligation of the two repaired *Sst*I termini (see Fig. 1).

Virus stocks were prepared by harvesting the medium from calcium phosphate-transfected FEK cells (30). Virus stocks were assayed by a micro reverse transcriptase (RT) assay (see below), and titers were determined in an infectivity assay based on an enzyme-linked immunosorbent assay (ELISA) (19). Briefly, 8 × 10⁴ FEK cells were mixed with various dilutions of virus-containing tissue culture supernatant and plated in triplicate in 24-well plates (Corning). Following overnight incubation at 37°C, an equal volume of medium containing 1.6% carboxymethyl cellulose (Sigma) was added, and the cells were incubated at 37°C for a total of 10 days. The plates were incubated at room temperature as follows: twice for 10 min each in 3.7% formaldehyde in phosphate-buffered saline (PBS) to fix cells; once for 30 min in 1% Triton X-100 in PBS to disrupt cells; once for 60 min in blocking buffer (1% fetal bovine serum in PBS); once for 60 min in primary antibody (anti-EIAV horse serum) diluted in blocking buffer; three times for 5 min each in wash buffer (0.05% Tween 20 in PBS); once for 60 min in horseradish peroxidase-conjugated goat anti-horse immunoglobulin G (United States Biochemical, Cleveland, Ohio) diluted in blocking buffer; and three times for 5 min each in wash buffer. The reaction was developed by incubation for 30 min at room temperature in 20 mM sodium acetate (pH 5.5) containing 0.19 mg of 3-amino-9-ethylcarbazole (Sigma) per ml, 0.57% dimethyl sulfoxide, and 0.023% H₂O₂. Titers (infectious-center doses [ICD]) were calculated by counting the number of infectious centers and multiplying this value by the reciprocal of the dilution factor.

Pony infections. Seronegative Shetland ponies were inoculated intravenously with 10^{5.0} ICD of EIAV. Rectal temperatures were monitored daily, and serum and plasma samples were taken at regular intervals.

Enzyme assays. dUTPase assays were performed as described previously (60). A micro RT assay was used to measure EIAV in clarified tissue culture supernatants. Clarified tissue culture supernatant (10 µl) was assayed in a 60-µl final volume that contained 83.3 mM Tris-HCl, 10 mM KCl, 8.3 mM MgCl₂, 8.3 mM dithiothreitol, 0.083% Nonidet P-40, 8.3 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 18.3 µg of poly(rA)·poly(dT)₁₂₋₁₈ (Pharmacia Biotech, Piscataway, N.J.) per ml, and 1.5 µCi of [*methyl*-³H]TTP (40 Ci/mmol; Amersham Life Sciences, Arlington Heights, Ill.). Following incubation at 37°C for 2 h, the entire sample was spotted onto a DE-81 filter disc (Whatman, Maidstone, England). Discs were dried under a heat lamp and then

washed three times in 1× SSC (15 mM NaCl, 1.5 mM sodium citrate) and once in 95% ethanol. After being dried as above, discs were counted in EcoLite (+) scintillation fluid (ICN, Costa Mesa, Calif.).

Quantitative RT-PCR. The virus present in plasma samples from experimentally infected ponies was pelleted through a 20% sucrose cushion in a Beckman SW 50.1 rotor at 120,000 × *g* for 45 min at 4°C. Virion RNA was extracted with Trizol reagent (GibcoBRL) as recommended by the manufacturer. Extracted RNA was precipitated in the presence of 1 µg of glycogen (Boehringer Mannheim, Indianapolis, Ind.) and then resuspended in a volume of H₂O such that a 100-fold concentration of the viral RNA was obtained. Reverse transcription of 2 µl of virion RNA was performed with the Superscript Preamplification System (GibcoBRL) as specified by the manufacturer, except that an EIAV *gag*-specific primer (*gag*34; 5' GCTGACTCTTCTGTGTATCG 3') was used at a final concentration of 0.4 µM to initiate first-strand cDNA synthesis. PCRs were carried out with a hot-start procedure, and the reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 0.05 mM each deoxynucleoside triphosphate (dNTP), 2% dimethyl sulfoxide, 0.3 µM *gag*34 primer, 0.3 µM *gag*11 primer (5' ATGTATGCTTGCAGAGACATTG 3'), 1 µCi of [*α*-³²P]dATP (3,000 Ci/mmol; DuPont NEN, Boston, Mass.), 1 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.), and 2 µl of cDNA in a final volume of 25 µl. PCR was carried out under the following cycling conditions: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles; 10 min at 72°C for 1 cycle; and hold at 4°C. As an external standard for quantitation of the viral RNA, RT-PCR was performed simultaneously on known amounts of a defined synthetic RNA template that contained an engineered 55-bp deletion of the *gag* gene. The synthetic RNA was prepared by in vitro transcription with a T7 MEGAScript kit (Ambion, Austin, Tex.). RT-PCR products were separated by electrophoresis in denaturing 6% acrylamide gels containing 8 M urea. Gels were fixed in a solution of 10% acetic acid and 10% methanol then dried. The radioactivity in each band was quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and the program Imagequant (Molecular Dynamics). A standard curve, based on the known amounts of synthetic RNA, was determined by linear regression analysis. The number of viral RNA molecules was then calculated by using the equation of the straight line.

Immunological assays. A peptide-based ELISA that measured antibody responses against the immunodominant peptides 12 and R32 of gp90 and gp45, respectively, was performed as described previously (1, 40). Antibodies against the native EIAV glycoproteins gp90 and gp45 were measured by a concanavalin A ELISA (19, 46). This assay did not measure antibodies directed against the EIAV core protein (p26), because the lectin does not capture nonglycosylated Gag proteins (19). Briefly, each well of a 96-well Immulon II plate (Dynatech Corp., Chantilly, Va.) was coated with 5 µg of concanavalin A followed by 1 µg of gradient-purified EIAV in 0.1% Nonidet P-40. After incubation with the pony serum samples, reactive antibodies were detected with goat anti-horse immunoglobulin G conjugated to horseradish peroxidase (United States Biochemical). All sera were tested at a 1:50 dilution. All ELISA results were quantitated on an MR5000 plate reader (Dynatech Corp.).

RESULTS

Construction of a deletion in the EIAV DU gene. The DU coding region of EIAV is located within the *pol* open reading frame, positioned between the RT and integrase genes (Fig. 1A). The predicted amino acid sequence of the EIAV DU protein shows a high degree of homology to the dUTPases of other nonprimate lentiviruses and to the human, yeast, and *E. coli* enzymes as well (58). Five conserved amino acid motifs present in all known dUTPase proteins have been recognized, and at least one of these motifs has been suggested to be functionally important (32). Motif 3 contains a highly conserved tyrosine residue, which has been suggested to be involved in catalysis (57). To construct an EIAV mutant that would be deficient in dUTPase activity, a *Sst*I restriction fragment containing 80% of the DU coding sequence, including four of the five conserved amino acid motifs, was deleted from the provirus clone PV19-2-6A (Fig. 1; see Materials and Methods). The deletion left intact the *pol* open reading frame and both protease-processing sites present on either side of the DU gene.

FEK cells were transfected with DNA prepared from the parental (PV 19-2-6A) and ΔDU mutant (PV 19-2-6A-ΔDU) EIAV provirus clones, and the tissue culture supernatant was assayed for RT activity as a measure of virus production. The results indicated that the DU gene was not essential for virus replication in FEK cells (Fig. 2). In fact, virus production was equivalent in FEK cells transfected with either the parental or

FIG. 1. Construction of a deletion within the DU gene of EIAV. (A) Genomic organization of EIAV. Below the genome map, an expanded DU gene is depicted. The positions of the two *SlyI* sites used to create the deletion are shown. The stippled bars represent the approximate positions of five conserved amino acid motifs present in all known dUTPase genes. LTR, long terminal repeat; PR, protease; IN, integrase. (B) Nucleotide and amino acid sequences flanking the two *SlyI* sites. The leucine residue is the first amino acid of the mature DU protein (45). A *pol* subclone containing the DU gene was digested with *SlyI*, and the resulting 5' termini were filled in with T4 DNA polymerase and ligated to generate the sequence shown below the arrow. The deleted DU gene was then inserted back into a full-length proviral clone (see Materials and Methods).

Δ DU mutant provirus clones, suggesting that deletion of the DU gene had no effect on the extent of EIAV replication. In addition, determination of the titer of virus stocks produced after transfection showed that the specific infectivities of the parental and Δ DU viruses, as measured in FEK cells, were very similar (data not shown).

To confirm that this deletion resulted in a virus deficient in dUTPase activity, virus harvested at 7 weeks posttransfection was assayed for dUTPase activity (Fig. 3). In contrast to the parental virus, the Δ DU mutant contained no detectable dUTPase activity.

FIG. 2. Transfection of parental and Δ DU proviral clones into FEK cells. FEK cells were transfected with 10 μ g of salmon sperm DNA (▲) or 10 μ g of PV 19-2-6A (●) or PV 19-2-6A- Δ DU (□) proviral DNA. Clarified tissue culture supernatants were assayed weekly for RT activity as described in Materials and Methods.

FIG. 3. dUTPase assay of parental and Δ DU viruses. Virus from clarified tissue culture supernatants was pelleted in an SW 28 rotor at $130,000 \times g$ for 2 h at 4°C. Virus pellets were resuspended in 50 mM Tris-HCl (pH 8.0)–1 mM dithiothreitol–20% glycerol, 250 mM KCl–0.5% Nonidet P-40 to disrupt the virus. Equivalent amounts of protein, as determined by the DC protein assay (Bio-Rad, Richmond, Calif.) were assayed for dUTPase activity. The negative control (no protein added to assay) was defined as 0% conversion. The positive control consisted of 1 μ g of disrupted gradient-purified EIAV. WT, wild type.

Replication kinetics in cultured cells. Since the efficiency of transfections in cell culture cannot be readily controlled, the replication kinetics of the parental and mutant virus stocks in FEK cells were examined more carefully. The multiplicity of infection (MOI) was varied to determine if the input inoculum had an effect on replication of the mutant virus. The mutant and parental viruses replicated with the same kinetics and to the same extent in FEK cells regardless of the MOI (Fig. 4), confirming that deletion of the DU gene had no effect on EIAV replication in this cell type.

Since dividing fibroblastic cells are not the natural targets for EIAV, we investigated the replication kinetics of parental and mutant viruses in cultures of primary (1°) EBMM cells (34). Primary EBMM cells were infected at various MOIs, and virus production was monitored by measuring RT activity in the culture supernatant. Detection of RT activity in the supernatant of Δ DU-infected 1° EBMM cells indicated that the DU gene is not required for replication in these cells (Fig. 5). However, several significant differences were observed between replication of the parental and Δ DU viruses. First, the Δ DU virus replicated to a lower level than the parental virus did. At an MOI of 1.0, 0.1, or 0.01 ICD per cell, there was approximately 10-, 50-, or 100-fold less virus in the Δ DU-infected cultures, respectively. Furthermore, the Δ DU-infected cells never achieved the peak levels of virus production seen in the cells infected with the parental virus. Second, the kinetics of virus accumulation were slightly slower in the Δ DU-infected cells than in the parental virus-infected cells (Fig. 5). Third, cells infected with the parental virus showed a decrease in virus production between 5 and 7 days postinfection (depending on the MOI) that was not seen in the Δ DU-infected cells (Fig. 5). Coincident with the peak of parental virus replication was the appearance of severe cytopathic effect, which consisted of cells rounding up and detaching from the tissue culture plate. For example, at 10 days postinfection at an MOI of 0.01 ICD per cell, most of the 1° EBMM cells infected with the parental virus had rounded up and detached from the tissue culture plate (Fig. 6B). Compared with uninfected cells, little or no such cytopathic effect was seen in Δ DU-infected 1° EBMM cells (Fig. 6A and C). At no time during the course of the experiment did any of the Δ DU-infected cultures appear significantly different from the uninfected controls, even at the highest MOI of 1.0 ICD per cell.

Replication in ponies. We investigated the replication and

FIG. 4. Replication kinetics of parental and Δ DU viruses in FEK cells. FEK cells (4×10^6 cells per well) were mock infected ($\blacktriangle$) or infected with parental ($\bullet$) or Δ DU ($\square$) virus. After 4 h at 37°C , the virus inoculum was removed and replaced with fresh medium. The medium was replaced daily. The harvested medium was centrifuged at $800 \times g$, and the supernatants were frozen at -70°C until assayed for RT activity. (A) MOI, 1 ICD per cell; (B) MOI, 0.1 ICD per cell; (C) MOI, 0.01 ICD per cell.

pathogenic properties of the Δ DU mutant virus in the Shetland pony model for EIAV infection. Infection of Shetland ponies with a defined inoculum of EIAV results in reproducible infections which are characterized by occurrence of the first detectable viremic episode 7 to 10 days postinfection and seroconversion between 14 and 21 days postinfection (34). When a pathogenic strain of virus is used, a disease episode usually occurs within 1 month of infection.

Shetland ponies were infected with approximately $10^{5.0}$ ICD per pony and monitored for fever as an indicator of disease and for virus levels in plasma and seroconversion as indicators of infection. At no time after infection did any of the ponies show signs of disease. Thus, as previously reported, the molecular clone of EIAV that was used was nonpathogenic (39), thereby precluding from these studies the evaluation of the role of DU in EIAV pathogenesis. However, this system did allow for detailed examination of the levels of virus replication and evaluation of the immune response upon infection with the Δ DU mutant.

To investigate the level of virus replication in vivo, levels of virus in plasma were measured by a quantitative RT-PCR assay. The results showed that although both viruses were able

FIG. 5. Replication kinetics of parental and Δ DU viruses in 1° EBMM cells. Primary EBMM cells (10^5 per well) were mock infected ($\blacktriangle$) or infected with parental ($\bullet$) or Δ DU ($\square$) virus. The cells were infected, and samples were harvested and assayed as in the experiment in Fig. 4. (A) MOI, 1.0 ICD per cell; (B) MOI, 0.1 ICD per cell; (C) MOI, 0.01 ICD per cell.

to replicate in vivo, the Δ DU-infected ponies had 10- to 100-fold-lower peak virus levels in plasma than did the parental virus-infected ponies (Table 1). Interestingly, the first detectable viremic episode occurred 5 to 10 days postinfection regardless of which virus was used to infect the animals. In addition, virus levels were generally higher in the parental virus-infected ponies throughout the course of infection. In fact, virus was often undetectable in the plasma of Δ DU-infected ponies. The sensitivity of the assay was determined to be between 30 and 50 molecules of RNA (data not shown). This indicates that even though the DU gene is not essential for virus replication in vivo, the presence of the gene imparts a significant replicative advantage to the virus.

The antibody response of each animal to native EIAV glycoproteins gp90 and gp45 was assessed by performing a concanavalin A ELISA. All of the ponies seroconverted between 14 and 42 days postinfection, within the normal time frame expected for an experimental infection (Fig. 7). Pony 76, which received the parental virus, made a strong response early after infection, which was maintained throughout the duration of the experiment (Fig. 7A). The antibody response of pony 52, which also received parental virus, was delayed and somewhat weaker (Fig. 7A). This pony made a strong response early after

FIG. 6. Cytopathic effect of parental and Δ DU viruses in 1° EBMM cells. Primary EBMM cells that had been infected for the experiment in Fig. 5 were photographed at 10 days postinfection. (A) Mock-infected cells; (B) cells infected with parental virus at an MOI of 0.01 ICD per cell; (C) cells infected with Δ DU virus at an MOI of 0.01 ICD per cell.

infection to linear epitopes present in gp90 and gp45 in a peptide-based ELISA (data not shown). Anti-glycoprotein antibody responses in the three ponies infected with the Δ DU virus were similar to those of the parental virus-infected animals (Fig. 7B). Antibody responses to whole virus, as measured by Western immunoblot analysis and agar immunodiffusion assay (Coggins test), and to the immunodominant peptides of gp90 (peptide 12) and gp45 (peptide R32), as measured by peptide ELISA, were slightly delayed and weaker in the Δ DU virus-infected ponies than in the parental virus-infected ponies (data not shown). Therefore, despite markedly reduced virus levels in plasma (Table 1), the Δ DU-infected ponies made

TABLE 1. Analysis of virus levels in plasma in experimentally infected ponies^a

Days postinfection	Amt of parental virus ^b in:		Amt of Δ DU virus in:		
	Pony 52	Pony 76	Pony 78	Pony 79	Pony 81
5	800	2,760	50	<30	50
6	460	2,920	<30	60	200
7	770	10,080	160	50	<30
8	3,110	1,210	130	190	<30
9	1,040	800	<30	130	<30
10	1,110	3,920	130	80	60
11	1,770	960	50	100	<30
12	1,980	610	60	70	<30
14	250	210	<30	<30	<30
15	330	180	<30	<30	<30
16	8,010	680	ND	<30	<30
17	340	2,450	<30	<30	<30
18	230	280	340	<30	60
19	860	210	<30	<30	<30
20	ND ^c	70	30	<30	40
21	100	100	<30	<30	<30
22	60	610	<30	<30	<30
35	<30	1,800	<30	<30	<30
40	<30	ND	<30	<30	<30
56	40	350	<30	<30	<30
63	<30	149	<30	<30	<30
75	5,890	330	<30	<30	<30
84	430	810	<30	<30	<30
90	270	260	<30	<30	80
97	180	160	<30	<30	<30
112	640	60	<30	<30	<30

^a Ponies were infected with $10^{5.0}$ ICD of parental or Δ DU virus. At the times indicated, plasma samples were prepared and viral RNA was purified, amplified, and quantitated as described in Materials and Methods.

^b Results are expressed as the number of molecules of RNA per 200 μ l of plasma. The limit of sensitivity of the assay was 30 molecules of RNA.

^c ND, not determined.

antibody responses similar to those observed for the ponies infected with the parental virus.

DISCUSSION

In this study, we examined replication of an EIAV deletion mutant deficient in dUTPase activity in two cell types in vitro and in vivo in a Shetland pony model of infection. Detailed examination of the replication kinetics showed that replication of the parental and Δ DU viruses was indistinguishable in FEK cells, but a significant decrease in Δ DU virus cytopathicity and a 10- to 100-fold decrease in virus production in 1° cultures of EBMM cells were observed (compare Fig. 4 and 5). Similar results were reported for a DU-deficient mutant of FIV and a different DU mutant of EIAV (54, 58). Two aspects of dUTPase regulation suggest that the different results seen in these two cell types may be a consequence of differential levels of endogenous dUTPase expression. First, dUTPase levels are high in dividing cells. Studies with proliferating T cells, regenerating rat liver, and dividing root meristems of higher plants all show a high degree of correlation between dividing cells and high levels of dUTPase activity (21, 37, 50). Second, regulation of dUTPase in a differentiation-dependent manner has been suggested. dUTPase activity was determined to be low in differentiated T cells, mature root and floral meristem cells, and adult (in contrast to newborn) rabbit brain and liver (37, 42, 49, 50). dUTPase was also shown to be developmentally regulated in *D. melanogaster* (15). Thus, actively dividing FEK cells may have an endogenous dUTPase activity which is sufficiently high

FIG. 7. Analysis of EIAV envelope glycoprotein-specific immune response. Antibodies directed against native EIAV envelope glycoproteins gp90 and gp45 were assayed by a concanavalin A ELISA procedure. All sera were diluted 1:50 before being assayed. (A) Serum from ponies infected with parental virus. Symbols: ■, pony 52; ▨, pony 76. (B) Serum from ponies infected with Δ DU virus. Symbols: ▩, pony 78; ▤, pony 79; ▥, pony 81. O.D. 490, optical density at 490 nm.

to compensate for the lack of virally encoded dUTPase activity in the Δ DU mutant. However, terminally differentiated non-dividing 1° EBMM cells may have low levels of endogenous dUTPase activity and thus cannot support efficient replication of the mutant in the absence of the virally encoded dUTPase. Support for this complementation model comes from in vitro studies of dUTPase mutants of HSV-1. Although infection of cells with wild-type HSV-1 results in down-regulation of cellular dUTPase expression, expression of cellular dUTPase remains normal in cells infected with dUTPase-deficient mutants of HSV-1 (12, 29, 59). Notably, wild-type and dUTPase-deficient mutants of HSV-1 replicate to the same extent in vitro (12, 43). As suggested by Lirette and Caradonna (29), the endogenous dUTPase compensates for the lack of virally encoded dUTPase activity such that replication of dUTPase-deficient HSV-1 remains normal. To determine the role of endogenous dUTPase in Δ DU virus replication, measurement of the endogenous levels of dUTPase in FEK and 1° EBMM cells is in progress.

Virus production was lower in Δ DU-infected 1° EBMM cells and never attained the peak level seen in cells infected with the parental virus (Fig. 5). This suggests that Δ DU virus replica-

tion was inhibited and not just delayed. This result may be attributed to either function of dUTPase, regulation of the dUTP/dTTP ratio, or synthesis of dUMP for use by thymidylate synthase. Control of the dUTP/dTTP ratio is an important function of dUTPase. In this regard, it has been suggested that the mechanism of thymineless death may not be due to the lack of dTTP available for DNA synthesis but to increased DNA damage as a consequence of an increased dUTP/dTTP ratio (17). Since DNA polymerases can utilize dUTP and dTTP equally well, an increase in the dUTP/dTTP ratio would result in inappropriate incorporation of uracil into DNA (13, 48). Repair of this lesion by uracil DNA glycosylase would create an apyrimidinic site, which would then be repaired by the sequential action of an endonuclease, DNA polymerase, and DNA ligase (55, 56). However, it is believed that a high dUTP/dTTP ratio during repair synthesis results in increased uracil incorporation into DNA, which leads to single- and double-strand breaks and ultimately to cell death (44). This mechanism is also important in the cytotoxic effect of chemotherapeutic drugs such as methotrexate, which inhibits folate metabolism, and 5-fluorothymidine, which inhibits thymidylate synthase (4, 5, 17).

Conversion of dUMP to dTMP by thymidylate synthase is a key step in the de novo synthesis of dTTP and of DNA. Decreased levels of dUTPase in Δ DU-infected 1° EBMM cells might adversely affect EIAV replication by reducing the pool of dTTP available for reverse transcription. As the abundance of dNTPs in human macrophages is very low, increasing the level of dNTPs may be an important factor in lentivirus replication in macrophages (41, 52). Interestingly, an increased rate of HIV (which does not encode a dUTPase) reverse transcription was achieved by supplementing macrophages with deoxynucleosides, although the effect was small (36). In addition, complete reverse transcription of Moloney murine leukemia virus in growth-arrested cells requires the exogenous addition of nucleosides to the cell medium (16). However, the importance of this pathway for the generation of dUMP and ultimately of dTTP may be limited. dUMP can also be synthesized by enzymatic deamination of dCMP, and this may represent the major pathway in eukaryotes. Also, the dTTP pool can be increased by using the well-characterized salvage pathway involving phosphorylation of thymidine by thymidine kinase.

As illustrated clearly for HIV and SIV *nef* mutants, it is essential to examine the phenotype of virus mutants in an appropriate animal model to accurately determine the function of a particular gene product. Conflicting results were initially reported when the replication of *nef* mutants was examined in vitro (11, 20, 26, 53). However, in vivo experiments showed a dramatically decreased replicative capacity of *nef* deletion mutants, demonstrating the critical role of Nef in SIV and HIV replication (23, 24). Therefore, replication of the Δ DU virus was examined in a Shetland pony model of EIAV infection. Similar results were obtained in vivo and in vitro in primary EBMM cells. The peak virus levels in plasma (as measured by a quantitative RT-PCR assay) in Δ DU-infected animals were 10- to 100-fold lower than virus levels in plasma of parental virus-infected ponies (Table 1). Furthermore, virus levels in plasma of Δ DU-infected ponies were often below the limit of detection (30 to 50 molecules). These results confirm the crucial role of dUTPase in replication that has been elaborated in other systems. Mutants of *E. coli* and *S. cerevisiae* totally lacking in dUTPase activity are not viable (10, 14). In a mouse model of infection, dUTPase mutants of HSV-1 were 10-fold less neurovirulent, approximately 1,000-fold less neuroinvasive, and less able to reactivate from latency than wild-type HSV-1 (43).

In spite of the low levels of virus in plasma, Δ DU-infected ponies made nearly normal antibody responses to the viral glycoproteins. This result is similar to the situation observed in monkeys infected with an SIV *nef* deletion mutant, in which virus replication was reduced 100-fold but immune responses to the mutant and wild-type viruses were similar (6, 24). These results, combined with the nonpathogenic nature of the SIV *nef* deletion mutant, led Kestler et al. (24) to suggest that *nef*-deleted lentiviruses could be used as live-virus attenuated vaccines. Although the Δ DU virus is clearly much less cytopathic in vitro (Fig. 6), the pathogenic potential of the Δ DU virus could not be assessed in vivo since the parental EIAV molecular clone itself is not pathogenic (39). The correlation between the in vitro and in vivo replication levels shown here and the relationship between high viral burden and EIAV pathogenesis (34) suggest that the Δ DU virus would not be expected to be pathogenic in vivo. Therefore, the ability of the Δ DU virus to protect animals from a virulent virus challenge is currently being investigated.

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