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Incorporation of Uracil into Viral DNA Correlates with Reduced Replication of EIAV in Macrophages

WENDY K. STEAGALL,*[1] MICHAEL D. ROBEK,*[2] STEPHANIE T. PERRY,† FREDERICK J. FULLER,† and SUSAN L. PAYNE*[3]

*Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106-4960; and †Department of Microbiology, Pathology and Parasitology, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina 27606

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The retrovirus equine infectious anemia virus (EIAV) encodes a dUTPase situated between reverse transcriptase and integrase. We have described the inability of EIAV with a 270-bp dUTPase deletion, ΔDU EIAV, to replicate to wild-type (WT) levels in equine macrophages (D. S. Threadgill, W. K. Steagall, M. T. Flaherty, F. J. Fuller, S. T. Perry, K. E. Rushlow, S. F. J. LeGrice, and S. L. Payne, *J. Virol.* 67, 2592–2600, 1993). Here we describe the construction of a second dUTPase-deficient virus (DU^{D71E}) containing a single amino acid substitution in dUTPase. ΔDU and DU^{D71E} replicate to 2% of WT levels in macrophages by 7 days postinfection, when WT EIAV is highly cytopathic. To identify the replication block(s), we analyzed DNA synthesis, integration, and transcription. DNA synthesis was normal in macrophages, with evidence of full-length viral DNA by 24 hr postinfection. The level of integrated ΔDU and DU^{D71E} DNA appeared to be decreased 2- to 3-fold compared to WT. Steady-state levels of full-length viral transcripts were decreased over 100-fold, indicating that replication of dUTPase-deficient EIAV is blocked between viral DNA synthesis and transcription. As dUTP hydrolysis normally plays a role in preventing incorporation of uracil into newly synthesized DNA, we investigated the possibility that dUTPase-deficient EIAV DNA contains uracil. *In vitro* assays showed that while WT virions do not utilize dUTP, dUTPase-deficient virus and recombinant RT synthesize uracil-containing DNA. The presence of uracil in viral DNA recovered from ΔDU- and DU^{D71E}-infected macrophages was also demonstrated. In macrophages, a virally encoded dUTPase may be necessary to prevent the incorporation of uracil into viral DNA. © 1995 Academic Press, Inc.

INTRODUCTION

Replication in nondividing cells is a hallmark of the lentivirus group of retroviruses. Unlike oncogenic retroviruses, which require host cell proliferation, or more specifically passage through mitosis, to integrate their viral DNA and to produce progeny, lentiviruses can productively infect cells arrested in the cell cycle (Bukrinsky *et al.,* 1992, 1993; Clements and Payne, 1994; Klevjer-Anderson *et al.,* 1979; Lewis and Emerman, 1994; Lewis *et al.,* 1992; Li *et al.,* 1993; Roe *et al.,* 1993; Varmus *et al.,* 1977; Weinberg *et al.,* 1991). The properties of lentiviruses that enable them to produce progeny in these cells have yet to be completely elucidated; however, studies on HIV-1 have implicated a viral core protein (Lewis *et al.,* 1992; Bukrinsky *et al.,* 1993) and have also uncovered active, ATP-based import of the HIV-1 preintegration complex into the nucleus, thus bypassing the need for the transient disassembly of the nuclear envelope upon passage through mitosis by the infected cell (Bukrinsky *et al.,* 1992). Similar mechanisms for replication in nondividing cells may be true of all lentiviruses, although similar studies have not yet been reported for the nonprimate lentiviruses. Some nonprimate lentiviruses, such as visna virus, EIAV, and feline immunodeficiency virus, have been shown to encode dUTPase activity (Clements and Payne, 1994; Elder *et al.,* 1992; Threadgill *et al.,* 1993; Wagaman *et al.,* 1993), and a study from our laboratory (Threadgill *et al.,* 1993) has indicated that this activity plays an important role in the replication of EIAV in nondividing macrophages.

dUTPases are ubiquitous cellular enzymes that play a key role in nucleotide biosynthesis. They promote the hydrolysis of dUTP, generating dUMP, a precursor in the dTTP biosynthetic pathway, and keep the cellular ratio of dUTP to dTTP low [less than or equal to 10^{-5} in mammalian cells (Richards *et al.,* 1986)]. As most DNA polymerases can utilize both dUTP and dTTP (Bessman *et al.,* 1958; El-Hajj *et al.,* 1988; Focher *et al.,* 1990; Mosbaugh, 1988; Shlomai and Kornberg, 1978), maintenance of the correct balance of deoxynucleotide pools, and of a low dUTP:dTTP ratio, is very important for fidelity in DNA repli-

[1] Present address: Department of Pediatrics, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106.

[2] Present address: Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110.

[3] To whom correspondence and reprint requests should be addressed. Fax: (216) 368-3055. E-mail: slp2@po.cwru.edu.

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cation. Uracil in DNA can arise either by incorporation of dUTP into nascent DNA or by cytosine deamination in mature DNA. Uracil is removed from DNA by uracil–DNA glycosylase (UDG), leaving an apyrimidinic site which is then repaired through the actions of apyrimidinic endonuclease, DNA polymerase, and DNA ligase. If the level of dUTP in the cell is high, a vicious circle of excision and repair of the DNA can result, leading to strand breaks, strand exchanges, and eventually cell death due to chromosomal aberrations and overlapping gaps in the DNA (Richards *et al.*, 1986). It has been demonstrated genetically in both prokaryotic (*Escherichia coli*) and eukaryotic (*Saccharomyces cereviseae*) systems that dUTPase is essential, as *dut* null mutants are inviable (El Hajj *et al.*, 1992; Gadsden *et al.*, 1993). *E. coli* with dUTPase mutations (*dut*$^-$ or *sof*$^-$) transiently accumulate small DNA fragments, probably due to the UDG-based repair process, and show a higher than normal frequency of recombination (Tye *et al.*, 1977, 1978); *dut*$^-$ *S. cereviseae* appear to become blocked in DNA synthesis. In repair-deficient genetic backgrounds (*udg*$^-$), both *dut*$^-$ *E. coli* and *dut*$^-$ *S. cereviseae* accumulate uracil in their DNA. The stable incorporation of high levels of uracil into DNA appears to cause a general failure in macromolecular biosynthesis (El Hajj *et al.*, 1992; Gadsden *et al.*, 1993). It has been demonstrated that uracil in DNA can affect DNA conformation and sequence-specific protein binding, as uracil lacks the methyl group of thymine (Dubendorff *et al.*, 1987; Focher *et al.*, 1992; Goeddel *et al.*, 1977; Richards *et al.*, 1986; Risse *et al.*, 1989; Verri *et al.*, 1990), and this may explain in part the lethality of stable uracil incorporation into DNA.

Expression of cellular dUTPases correlates with the state of cellular differentiation: i.e., high in undifferentiated, dividing cells and low in terminally differentiated, nondividing cells (Duker and Grant, 1980; Mahagaokar *et al.*, 1980; Pardo and Gutierrez, 1990; Pri-Hadash *et al.*, 1992; Spector and Boose, 1983). Thus, a virally encoded dUTPase activity could assist in the establishment of viral replication in nondividing host cells. We deleted a portion of the dUTPase gene in equine infectious anemia virus (EIAV) and found that dUTPase function appears to be necessary for efficient viral replication in macrophages (Threadgill *et al.*, 1993), as this dUTPase-deleted virus (ΔDU) replicates poorly [less than 1% of wild-type (WT) levels] in equine macrophages, the natural host cell of the virus, while replicating to WT levels in dividing cells. In the present studies, we describe the growth phenotype of a second dUTPase-deficient virus (DU^{D71E}), which contains a point mutation in the proposed active site of dUTPase, and present an investigation of the block(s) to virus replication in macrophages. We have looked at several steps in viral replication that could be affected by the loss of dUTPase activity, including viral DNA synthesis, integration, and transcription.

MATERIALS AND METHODS

Cells and viruses

Feline embryonic adenocarcinoma (FEA) cells and equine macrophages were prepared and maintained as described previously (Threadgill *et al.*, 1993). Conditions for FEA cell transfection and the preparation of virus stocks have also been described (Threadgill *et al.*, 1993).

Mutagenesis

A *Kpn*I–*Pst*I fragment containing the dUTPase coding domain from the proviral clone pSPEIAV19 (Payne *et al.*, 1994) was subcloned into pBS+ (Stratagene), and a point mutation introduced using the Doubletake Double-Stranded Mutagenesis kit (Stratagene) following the manufacturer's suggested protocol. The mutagenic primer for the DU^{D71E} mutation is 27-mer of the sequence 5′-TGTATATCCTTCTTCAATTATTCCTCC-3′ (noncoding strand), with the base change in boldface [a T to A change in the coding strand at base number 4120 of the EIAV proviral sequence (GenBank Accession Nos. M16575, M11337, K03334, and M14855)]. This represents an aspartic to glutamic acid substitution at amino acid number 71 of the dUTPase amino acid sequence. DNA sequence analysis confirmed the presence of the mutation, and the mutated dUTPase region was substituted into a plasmid containing the 5′ end of pSPEIAV19 (Threadgill *et al.*, 1993). This mutated 5′ end was then ligated via a unique *Sph*I site to DNA derived from the 3′ end (*Sph*I to *Eco*RI) of pSPEIAV19, and the ligation mix used to transfect FEA cells.

dUTPase assays

dUTPase assays were performed as described previously (Threadgill *et al.*, 1993). Reaction mixes contained, in a 0.1-ml reaction volume, 50 m*M* Tris–HCl (pH 7.4), 2 m*M* β-mercaptoethanol, 1 m*M* $MgCl_2$, 0.1% wt/vol bovine serum albumin, 2 m*M* *p*-nitrophenylphosphate, and a total of 0.1 m*M* dUTP ([^{3}H]dUTP at 50 μCi/μmol). Reactions were incubated at 37° for 15 to 30 min.

PCR analysis of viral DNA

WT and ΔDU viral stocks were treated with 2 μg/ml RNase-free DNase (Worthington) to remove any DNA from lysed cells in the stocks that could confound results obtained with the polymerase chain reaction (PCR). FEA cells were incubated with 1×10^7 cpm of virus (as measured by reverse transcriptase activity) in the presence of 10 μg/ml polybrene. After 1 hr at 37°, the virus was removed and the cells were washed three times followed by addition of fresh media. Equine macrophages were incubated with 5×10^5 cpm of virus (multiplicity of infection of about 1) for 1 hr at 37°, washed, and refed. Following the methods of Zack *et al.* (1990; see also Arrigo *et*

TABLE 1

Oligonucleotide Sequences

Oligonucleotide	Sequence
209	5'-GAGTCCTTCTTGCTGGGCTGAAAAG-3'
351	5'-CCCCCCCCGTCGACGGTAGGGTCTGCGCC-3'
3439	5'-TTGGCTCCCAGAAATAGTATATACA-3'
4519	5'-GCATGTATGTATCCTGAATTTGAC-3'
TNF A	5'-GCAAACCTTAAGGGTCTCAC-3'
TNF B	5'-GCAAGGGCTCTTGATGGCAG-3'

al., 1989, and Zack *et al.,* 1992), cells were lysed in urea lysis buffer (4.7 *M* urea, 1.3% SDS, 0.23 *M* NaCl, 0.67 m*M* EDTA, pH 8.0, 6.7 m*M* Tris–Cl, pH 8.0) at various times. Lysates were extracted with phenol:chloroform (25:24:1 phenol:chloroform:isoamyl alcohol) and ethanol precipitated. PCR reactions included approximately 1.8 μg DNA (amounts of DNA were first normalized by PCR amplification of a portion of the α-globin gene; data not shown), 0.25 m*M* dNTPs, 50 m*M* NaCl, 25 m*M* Tris–Cl, pH 8.0, 5 m*M* $MgCl_2$, 100 μg/ml bovine serum albumin, 50 ng oligo 351 (Table 1), 30 ng 5′ ^{32}P-labeled oligo 209 (Table 1), and 2.5 units *Taq* DNA polymerase (Boehringer-Mannheim). Twenty-five cycles of PCR were performed (1 min at 91°, 2 min at 60°, for annealing and extension). Products were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels, followed by autoradiography.

Reverse transcriptase assays

Reverse transcriptase (RT) assays to quantitate viral replication in FEA cells and in macrophages have been described previously (Threadgill *et al.,* 1993). In RT assays using pelleted virus, culture supernatants were clarified by low-speed centrifugation and virus was collected by centrifugation through 20% glycerol at 25,000 rpm for 2 hr in an SW28 rotor. Viral pellets were resuspended in 50 m*M* Tris–Cl, pH 8.0, and protein concentrations measured using the Bio-Rad protein assay reagent. Exogenous RT assays contained 20 ng recombinant EIAV RT (LeGrice *et al.,* 1991) or 0.4 mg/ml pelleted virus, 0.25 optical density unit of poly(rA) poly$(dT)_{12-18}$, 50 m*M* Tris–Cl, pH 8.0, 7.5 m*M* KCl, 2 m*M* DTT, 5 m*M* $MgCl_2$, 50 μM dUTP or dTTP, and 1.52 μM [^{3}H]dUTP or [*methyl*-^{3}H]dTTP. Assays containing pelleted virus also contained 0.05% NP-40 and 0.02% Triton X-100. Reactions were incubated at 37° for 1 hr, and two aliquots were spotted on DE81 paper. One aliquot was washed twice in 2× SSC (1× SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate), twice with 95% ethanol, and dried. Samples were counted in the presence of scintillation fluid (Cytoscint). Competition RT reactions contained 1.52 μM [^{3}H]dUTP and a total of 52 μM dUTP plus dTTP.

Endogenous RT assays contained 0.5 mg/ml virus, 0.1 *M* Tris–Cl, pH 8.0, 5 m*M* magnesium acetate, 50 m*M* NaCl, 10 m*M* DTT, 0.02% Triton X-100, 200 μM each dATP, dCTP, dGTP, and 38 μM dUTP or dTTP, and 1.52 μM [^{3}H]dUTP or [*methyl*-^{3}H]dTTP (Rice and Coggins, 1979). Reactions were incubated at 42° for 1 hr and then treated as above. Competition RT reactions contained 1.52 μM [^{3}H]dUTP and a total of 40 μM dUTP plus dTTP.

Exogenous RT assays in which recombinant dUTPase (Robek *et al.,* manuscript in preparation) was added *in trans* were performed essentially as described above except that the level of dUTPase activity present in WT virions was determined and recombinant dUTPase (in amounts yielding the units of activity indicated in Fig. 3C) was added to RT assays containing ΔDU virus or recombinant RT (rRT).

Uracil–DNA glycosylase assays

Infected equine macrophages were lysed in extraction buffer (10 m*M* Tris–Cl, pH 8.0, 0.1 *M* EDTA, pH 8.0, 0.5% SDS) at various times postinfection and DNA was prepared by the method of Sambrook *et al.* (1989). UDG treatment and PCR were performed in reactions containing 1 to 2 μg DNA, 0.25 m*M* dNTPs, 25 m*M* NaCl, 25 m*M* Tris–Cl, pH 8.0, 5 m*M* $MgCl_2$, 100 μg/ml bovine serum albumin, 1 m*M* DTT, 20 pmol each of EIAV polymerase region primers 3439 and 4519 (Table 1), and 1.0 to 2.5 units uracil–DNA glycosylase (UDG) (Boehringer-Mannheim or Perkin–Elmer). Primers to amplify a portion of the equine tumor necrosis factor (TNF) gene (TNF A and TNF B; Table 1) were added to provide an internal control for PCR. Reactions were incubated for 1 hr at 37° and 5 min at 95° for UDG treatment and DNA cleavage, followed by the addition of 2.5 units of *Taq* DNA polymerase for initiation of PCR. Products of the reaction were analyzed by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. In some experiments Southern blot analysis was performed to confirm the identity of the PCR products (data not shown). UDG treatment and amplification of DNA from infected FEA cells were performed as described above except that 30 to 50 ng of ^{32}P-labeled 3439 and 4519 primers were used in each reaction, and equivalent amounts of PCR products were analyzed by electrophoresis on 4% nondenaturing polyacrylamide gels.

Southern blot analysis

Macrophage or FEA cell DNA, prepared as described above, was fractionated by electrophoresis on 0.7% agarose gels and transferred to nitrocellulose by standard methods (Sambrook *et al.,* 1989). Membranes were subjected to uv-crosslinking, baked at 80°, and prehybridized for 1–2 hr in 6× SSPE, 10× Denhardts (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 1% SDS,

and 50 μg/ml denatured salmon sperm DNA. Blots were hybridized overnight in 6× SSPE, 1% SDS, 10% dextran sulfate, 100 μg/ml denatured salmon sperm DNA, and 2 × 10^6 cpm/ml of a ^{32}P-labeled probe generated by random primed synthesis of portions of a full-length EIAV proviral clone. Membranes were washed as follows: two times for 15 min at room temperature in 6× SSPE, 0.5% SDS; two times for 15 min at 37° in 1× SSPE, 0.5% SDS; and once for 20 min at 68° in 0.1× SSPE, 0.5% SDS, followed by autoradiography.

RNA preparation and Northern blot analysis

RNA was prepared either by the cesium chloride centrifugation method described by Sambrook *et al.* (1989) or by the method of Chomczynski and Sacchi as modified by Xie and Rothblum (Chomczynski and Sacchi, 1987; Xie and Rothblum, 1991). In the latter method, infected FEA cells or macrophages were lysed in 1.9 *M* guanidinium thiocyanate, 11.9 m*M* sodium citrate, pH 7.0, 95 m*M* sodium acetate, pH 4.0, and 0.34% β-mercaptoethanol. Water-saturated phenol and chloroform:isoamyl alcohol (24:1) were added to 47.5 and 10%, respectively, and lysates were vortexed and placed on ice for 20 min. Nucleic acid in the aqueous layer was precipitated with an equal volume of isopropanol, washed twice with 70% ethanol, and resuspended in DEPC-treated dH_2O.

Samples were fractionated by electrophoresis in formaldehyde-containing agarose gels in 1× MOPS [0.02 *M* MOPS (3-[*N*-morpholino]propanesulfonic acid), 5 m*M* sodium acetate, 1 m*M* EDTA, pH 7.0] with buffer circulation. The gels were treated with 0.05 *N* NaOH for 30 min and 0.1 *M* Tris–Cl, pH 8.0, for 30 min and transferred to nitrocellulose in 10× SSC. The membranes were subjected to uv-crosslinking, baked at 80°, prehybridized in 1% bovine serum albumin, 0.4 *M* sodium phosphate, pH 7.2, 15% formamide, 1 m*M* EDTA, pH 8.0, and 7% SDS, pH 7.2, at 65° for 1–2 hr, and hybridized overnight at 65° with 3 × 10^6 cpm of probe. Probes used were either a ^{32}P-labeled actin fragment generated by random primed synthesis or a single-stranded DNA probe generated by runoff PCR in the presence of a [^{32}P]dCTP. This PCR-generated probe hybridizes to the region of the RNA 5′ to the major splice donor site, thereby hybridizing to all EIAV mRNAs. Membranes were washed 30 min at 50° in 2× SSC, 1% SDS, 30 min at 50° in 0.2× SSC, 0.1% SDS, and 30 min at 65° in 0.2× SSC, 0.1% SDS, followed by autoradiography.

RESULTS

We have examined several stages in the EIAV life cycle that might be affected by the loss of dUTPase activity, including reverse transcription, integration, and transcription, using two dUTPase-deficient viruses. ΔDU EIAV, which contains a 270-bp deletion in the dUTPase domain, has been previously described (Threadgill *et al.*, 1993). The second virus, DU^{D71E}, contains a single amino acid substitution in the dUTPase domain and was constructed to confirm that the phenotype described for the deletion mutant was in fact due solely to the loss of dUTPase activity. In the current studies, we have compared replication of WT and dUTPase-deficient viruses in macrophages during the initial round(s) of infection and prior to the onset of cytopathic effects in the WT EIAV-infected cultures. Replication of both WT and dUTPase-deficient EIAV was also characterized in the dividing feline adenocarcinoma cell line, FEA, in which dUTPase-deficient and WT viruses replicate to equivalent titers.

TABLE 2

Viral dUTPase Activity

Virus	dUTPase activity[a] (% conversion)
Wild type	96.0
ΔDU	17.0
DU^{D71E}	20.5

[a] Values represent the percentage conversion of dUTP to dUMP plus PP_i and are the averages of two to three experiments.

Preparation and analysis of DU^{D71E} EIAV

Site-directed mutagenesis was used to create an aspartic acid to glutamic acid substitution at amino acid number 71 of the EIAV dUTPase protein. Based on the crystal structure of the *E. coli* dUTPase (Cedergren-Zeppezauer *et al.*, 1992), this aspartic acid is predicted to be in the active site of the enzyme. dUTPase D71E was expressed in *E. coli*, purified, and the enzymatic activity of the mutant protein was assayed in standard dUTPase assays. We were unable to detect dUTPase activity at protein levels up to 100 times those used in assays of WT recombinant EIAV dUTPase in our laboratory (data not shown), indicating the critical nature of aspartic acid 71 for enzyme activity (Robek *et al.*, manuscript in preparation). dUTPase D71E was then used to replace the dUTPase domain of the infectious clone pSPEIAV19, and DNA containing the mutation was transfected into FEA cells for the production of viral stocks. As shown in Table 2, neither ΔDU nor DU^{D71E} EIAV have significant dUTPase activity compared to WT virus.

DU^{D71E} EIAV was then used to infect FEA cells to examine its replication phenotype and to obtain virus stocks for macrophage infections. As seen in Fig. 1A, both dUTPase-deficient viruses replicate to WT levels in FEA cells. In contrast, as shown in Fig. 1B, the production of the dUTPase-deficient viruses is only 2% of WT levels at 7

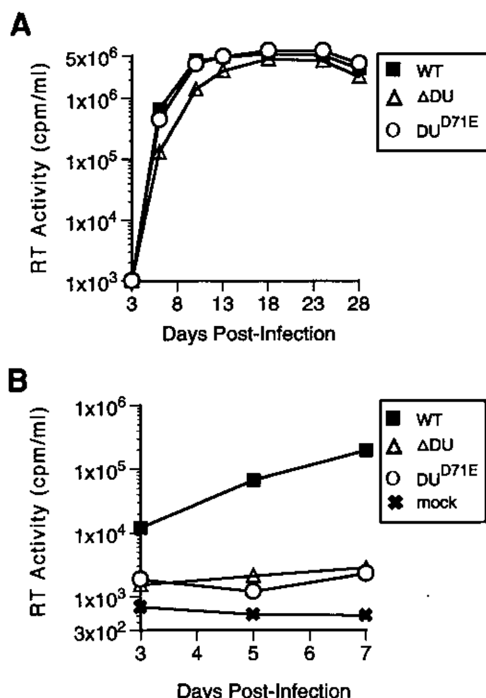

FIG. 1. Growth curves of WT- and dUTPase-deficient EIAV in FEA cells (A) and primary equine macrophage cultures (B). Equivalent amounts (based on RT units) of each virus stock were used for infections. Virus replication was monitored by RT assay.

days postinfection, when WT cultures exhibit maximum cytopathic effects.

Viral DNA synthesis

The kinetics of viral DNA synthesis by WT and ΔDU viruses were examined in both FEA cells and macrophages to determine if the replication defect of dUTPase-deficient EIAV in macrophages could be attributed to a block in viral DNA synthesis. Cells were incubated with equivalent amounts of virus, as measured by reverse transcriptase activity, and lysed at various times postinfection. PCR was then used to analyze the progress of viral DNA production, using a primer pair (209 and 351; see Table 1 and Fig. 2A) designed to amplify only full-length or nearly full-length viral DNA. As shown in Fig. 2A, full-length DNA can be detected by PCR analysis at 4 hr postinfection of FEA cells; no reproducible differences in viral DNA synthesis were observed between the WT and ΔDU viruses. In macrophages the kinetics of DNA synthesis appear to be slower, with synthesis of full-length viral DNA occurring between 6 and 24 hr postinfection. In contrast to FEA cell infections, where approximately equivalent amounts of PCR product were obtained for WT and ΔDU, we repeatedly see an increase in the amount of PCR product obtained from ΔDU-infected macrophages. Similar results were obtained using other primer pairs and following normalization of input DNA by PCR amplification of a portion of the α-globin gene (data not shown), indicating that ΔDU viral DNA synthesis may be more efficient than WT in macrophages. While we cannot rigorously rule out a higher quantity of input ΔDU virus in the experiment shown, this explanation seems unlikely as we have seen similar results with the use of different virus stocks. We also fail to observe reproducible differences in PCR product accumulation in infected FEA cells. An alternative explanation is that the ability of ΔDU virions to utilize dUTP as well as dTTP for DNA synthesis (see Fig. 3) could facilitate viral DNA production when deoxynucleotide pools are low, as might be encountered in nondividing macrophages (O'Brien *et al.,* 1994).

Viral DNA synthesis in macrophages was also examined directly by Southern blot analysis. DNA was obtained from WT, ΔDU, and DU^{D71E} EIAV-infected macrophages at 48 to 72 hr postinfection. Undigested DNA was analyzed by electrophoresis on 0.7% agarose gels, transferred to nitrocellulose membranes, and probed with EIAV-specific probes. As shown in Fig. 2B, full-length unintegrated linear EIAV DNA is apparent at 48 or 72 hr postinfection for all viruses; similar results have been obtained as early as 24 hr postinfection (data not shown). The results from both PCR and Southern blot analyses reveal that the replication block of dUTPase-deficient viruses in macrophages occurs at a step after the synthesis of viral DNA.

Detection of uracil in viral DNA

Following the observation that dUTPase-deficient viruses efficiently synthesize viral DNA, we sought to determine if EIAV RT could utilize uracil in the presence or absence of dUTPase. Both *in vitro* and *in vivo* studies were performed. First we examined the ability of EIAV RT to incorporate uracil into DNA products in both exogenous and endogenous RT assays. In the exogenous reaction, pelleted virus or recombinant EIAV RT were incubated with poly(rA) poly$(dT)_{12-18}$, and either [^{3}H]dUTP or [*methyl*-^{3}H]dTTP. In the endogenous reactions, pelleted virions were utilized both as a source of RT and of template RNA. As shown in Fig. 3A, ΔDU EIAV is capable of incorporating either dUTP or dTTP into DNA products, while the WT virus does not efficiently utilize dUTP. Also shown in Fig. 3A, recombinant EIAV RT, similar to other DNA polymerases (El-Hajj *et al.,* 1988; Focher *et al.,* 1990; Mosbaugh, 1988), utilizes dUTP. Figure 3B shows competition experiments in which ΔDU virions were incubated with [^{3}H]dUTP and varying ratios of dUTP to dTTP. These results indicate that in the absence of an active dUTPase, virion-associated RT does not show a strong preference for dTTP vs dUTP. To quantitate the ability of recombinant EIAV RT to utilize dUTP vs dTTP, we determined the Kms for each substrate using a poly(rA)

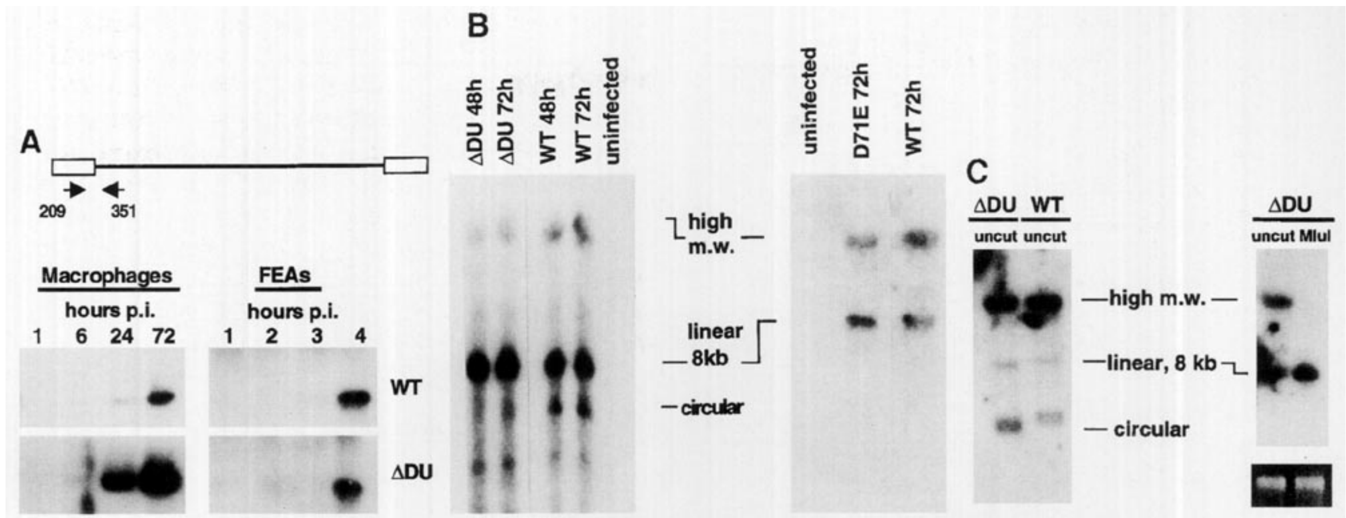

FIG. 2. Analysis of DNA synthesis of ΔDU and WT EIAV in macrophages and FEA cells. (A) Primers 209 (5′ ^{32}P-labeled) and 351 were used for PCR amplification of DNA recovered from macrophages or FEA cells at the indicated times postinfection. Primers 209/351 amplify only completed or nearly completed double-stranded viral DNA. PCR reaction products were analyzed by electrophoresis on nondenaturing polyacrylamide gels followed by autoradiography. (B) Southern blot analysis of viral DNA from equine macrophages. Samples of total DNA recovered (at the indicated times postinfection) from uninfected cells or cells infected with ΔDU, DU^{D71E}, or WT EIAV were electrophoresed on 0.7% agarose gels, transferred to nitrocellulose membranes, and probed with ^{32}P-labeled probes as described under Materials and Methods. Blots from two independent infections comparing ΔDU and WT, and DU^{D71E} and WT, are shown. (C) Southern blot analysis of total DNA from persistently infected FEA cells. DNA samples were untreated or digested with restriction enzyme *MluI* as indicated. A portion of the ethidium bromide-stained gel, showing the high-molecular-weight DNA, is shown directly beneath the autoradiogram. The stained gel shows little evidence of chromosomal DNA digestion by *MluI*.

poly$(dT)_{12-18}$ template. The calculated Kms for the recombinant enzyme were 15 and 20 μM for dUTP and dTTP, respectively.

Finally, we determined the level of dUTPase present in WT virions and performed a reconstitution experiment by the addition of recombinant dUTPase to ΔDU virions. As shown in Fig. 3C, we see little incorporation of dUTP into DNA products with the addition of recombinant dUTPase at levels 5- to 40-fold lower than those found in WT virions. Similarly, if we add 50 units of recombinant dUTPase to reactions containing recombinant EIAV RT, we see little utilization of dUTP (Fig. 3C). Taken together, this group of experiments suggests that the dUTPase activity present in WT EIAV keeps local concentrations of dUTP low so that this deoxynucleotide is not available for utilization by RT, which does not show a strong preference for dTTP as compared to dUTP.

After determining that ΔDU virions incorporate uracil into DNA *in vitro,* we examined the viral DNA recovered from infected macrophages for the presence of uracil. DNA samples were incubated with UDG, which specifically removes the uracil base from DNA, leaving apyrimidinic sites. The samples were then heated, causing strand breaks at the apyrimidinic sites, and PCR was performed to see if amplification of EIAV DNA was abrogated by this treatment. A region of the polymerase gene, flanking the dUTPase region, was amplified; the WT and DU^{D71E} amplification products are 1103 bp while the ΔDU amplification product is 833 bp, reflecting the 270-bp deletion of the DU region. A portion of the equine TNF gene was also amplified in each reaction as an internal control for UDG treatment and amplification efficiency. As shown in Fig. 4, the ability to amplify EIAV sequences from ΔDU- or DU^{D71E}-infected macrophages decreases following UDG treatment (compare the amount of viral-specific product in each lane with the internal TNF control). In contrast, there is little decrease in the EIAV-specific product obtained from UDG-treated WT-infected macrophages. The above experiments were performed using five different DNA sets with similar results (data not shown).

Figure 4B presents another set of control experiments for UDG treatment that was performed using DNA from WT- or ΔDU-infected FEA cells, in which these viruses replicate to equivalent titers. PCR was performed using ^{32}P-labeled primers, and equivalent amounts of each reaction were analyzed by gel electrophoresis and autoradiography. The TNF internal control was not used for the FEA samples as the primers are based on equine sequence and do not amplify feline DNA. In these experiments, in contrast to the results observed with macrophages, accumulation of viral-specific PCR products was similar for ΔDU and WT infections, although the total amount of PCR product appears to decrease with UDG treatment of either sample. The results of the UDG treatment experiments described above, although not quantitative, strongly suggest that uracil is present in viral DNA

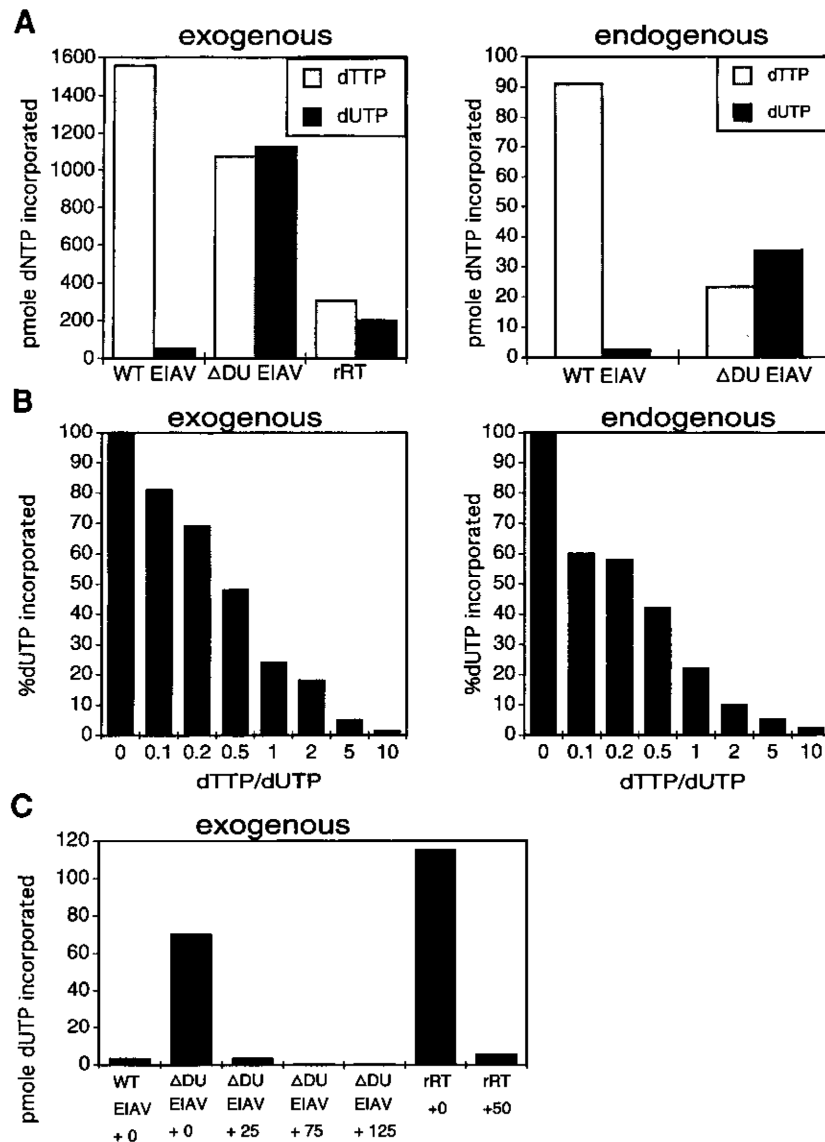

FIG. 3. (A) Exogenous and endogenous RT assays to determine utilization of dUTP by EIAV RT. ΔDU or WT virions, or recombinant EIAV RT, were incubated with ^{3}H-labeled dUTP or dTTP and either a poly(rA) poly(dT)$_{12-18}$ substrate (exogenous reaction) or the viral RNA template (endogenous reaction). (B) Competition reactions to determine if virion-associated RT has a preference for dTTP vs dUTP. ΔDU virions were incubated with templates (as in A) and various ratios of dUTP to dTTP. (C) Reconstitution experiments in which RT reactions containing WT or ΔDU virions or 20 ng of recombinant RT (rRT) were supplemented with up to 125 units of recombinant EIAV dUTPase as indicated. The WT virus sample contained approximately 1000 units of virion-associated dUTPase activity.

products synthesized by dUTPase-deficient viruses in macrophages.

Integration

As it is possible that viral DNA containing uracil would be subject to host repair mechanisms that would prevent integration, we attempted to quantitate the amount of integrated provirus found in macrophages early after infection by analysis of Southern blots of macrophage DNA. The blots in Fig. 2B show a specific hybridization signal in the region of high-molecular-weight chromosomal DNA (compare EIAV-infected to uninfected lanes). Upon examination of blots from several infections, we routinely observed slightly less signal in the high-molecular-weight region for DNA from ΔDU-infected macrophages. Using densitometry, the amount of high-molecular-weight signal (integrated viral DNA) was compared to that of the unintegrated linear DNA present in each sample. The ratio of the signal corresponding to high-molecular-weight DNA to the signal corresponding to unintegrated viral DNA was an average of 2.4 times lower with the ΔDU virus than with the WT (with five sets of ΔDU and WT DNA analyzed). Similar results were ob-

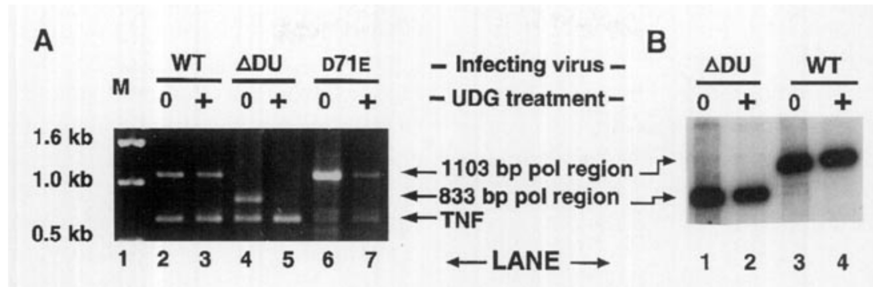

FIG. 4. Uracil–DNA glycosylase (UDG) analysis of viral DNA from macrophages (A) and FEA cells (B). DNA from infected cells was treated with UDG as described under Materials and Methods and amplified with primers 3439 and 4519. The ΔDU amplification product is 833 bp vs 1103 bp for WT and DU^{D71E}, reflecting the 270-bp deletion in the dUTPase region. Primers TNF A and TNF B were added to the reactions to provide internal controls for quantity of input DNA, UDG treatment, and amplification.

tained in two DU^{D71E} infections. In contrast, as shown in Fig. 2C, when persistently infected FEA cells were used as a source of DNA, no difference was observed in the amount of EIAV sequences present in the high-molecular-weight region of the gel. The EIAV-specific hybridization signals seen in the high-molecular-weight regions of the gels in Figs 2B and 2C likely represent integrated provirus. Although we can not rigorously rule out that this hybridization is due to trapped unintegrated viral DNA, digestion of FEA cell DNA with *Mlu*I, which cuts once within each viral LTR but which cuts chromosomal DNA very rarely, converts most of the signal present in the high-molecular-weight DNA region to an 8-kb linear species (see Fig. 3C). As shown in Fig. 3C, ethidium bromide staining reveals that *Mlu*I treatment does not decrease the size of the bulk of the chromosomal DNA. Due to low recoveries of DNA from macrophages, we used a PCR-based assay (based on the assay in Lewis *et al.,* 1992, and Lewis and Emerman, 1994) to detect integrated viral DNA in these cells and were able amplify PCR products reflective of integrated proviral DNA (data not shown). Therefore, ΔDU and DU^{D71E} appear to integrate their DNA during macrophage infection, although somewhat less efficiently than WT.

Transcription

Steady-state levels of viral RNA were examined by Northern blot analysis, as it is possible that the presence of uracil in viral DNA, or the disruption of proviral sequences via host-mediated repair processes during or after integration, might result in decreased production of viral transcripts. RNA was prepared from infected macrophages at 72 hr postinfection and from persistently infected FEA cells, separated by electrophoresis on formaldehyde-containing agarose gels, and probed with actin- and viral-specific probes. As shown in Fig. 5, viral transcripts are detected in both cell types; however, the steady-state levels of the ΔDU transcripts are decreased compared to WT. Quantitation by densitometry performed on a Northern blot from macrophages (shown in Fig. 5A) indicates about a 25-fold decrease in the levels of ΔDU multiply spliced messages compared to those of WT. Levels of singly spliced and full-length messages are even more severely decreased (approximately 85- and 300-fold, respectively; all samples were normalized by comparison of actin signals). This is a minimum estimate, as other Northern blots [including two performed with DU^{D71E} (data not shown)] do not show detectable levels of viral transcripts although the amount of actin hybridization in these samples is comparable to that seen with

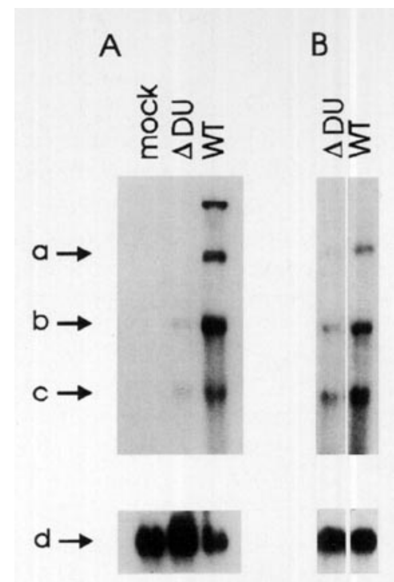

FIG. 5. Northern blot analyses. (A) RNA recovered from uninfected and ΔDU- or WT-infected macrophages at 72 hr postinfection. (B) RNA recovered from persistently infected FEA cells. Viral transcripts were detected using a single-stranded viral-specific probe as described under Materials and Methods. The labeled viral bands are: (a) full-length viral RNA, (b) singly spliced viral RNA, (c) multiply spliced viral RNA, and (d) actin mRNA. The unlabeled high-molecular-weight bands in A correspond to the position of the wells and represent RNAs retained in the wells.

RNA from WT-infected macrophages. Quantitation by densitometry of viral transcripts from persistently infected FEA cells indicates only a 3-fold difference in levels of ΔDU messages as compared to those of WT (in analysis of two sets of RNA samples; see Fig. 5B). The decrease in the steady-state levels of viral transcripts observed in macrophages infected with dUTPase-deficient viruses could account for the 100-fold decrease in virus production in this cell type.

DISCUSSION

DU^{D71E}

EIAV encodes a dUTPase whose activity has been previously shown to be important for replication in equine macrophages, the natural host cell for this virus (Threadgill *et al.*, 1993). Another dUTPase-defective virus, DU^{D71E}, has been created to expand our studies of EIAV dUTPase, and the block(s) to the replication of dUTPase-deficient viruses in macrophages has been investigated. The DU^{D71E} virus was constructed to address concerns that the replication phenotype of ΔDU EIAV in macrophages was not due solely to the lack of dUTPase activity, but might be an artifact of the deletion itself, such as an unforeseen effect on integration, splicing, or polyprotein processing. In DU^{D71E} EIAV, the replacement of an aspartic acid with a glutamic acid in the putative dUTPase active site was the result of a single nucleotide substitution, making it unlikely to result in unforeseen secondary effects on virus replication. As the replication of both ΔDU and DU^{D71E} EIAV are essentially identical in macrophages, we conclude that the loss of dUTPase activity itself is the most likely explanation for poor replication of these viruses. The introduction of exogenous dUTPase into macrophages to complement dUTPase-deficient EIAV would confirm our conclusions; however, due to the low transfection efficiency of macrophages, this experiment is unfeasible at this time.

Production of viral DNA

As a first step toward elucidating the replication block(s) of dUTPase-deficient viruses in macrophages, the synthesis of viral DNA was examined by PCR and Southern blot analysis. Full-length viral DNA can be detected in FEA cells by 4 hr and in macrophages by 24 hr following infection with either WT or ΔDU EIAV. Thus a block to viral DNA synthesis is not the cause for the observed difference in replication efficiency of these two viruses in macrophages. These experiments do reveal a difference in the rate of accumulation of viral DNA in FEA cells vs macrophages, however. This difference is probably not due to the quantity of input virus, as a slightly higher multiplicity of infection was used with macrophages than with FEA cells. Instead, the results may reflect differences in deoxynucleotide pools between actively dividing (FEA) and highly differentiated nondividing (macrophage) cells. Studies on HIV-1 replication in macrophages indicate that production of viral DNA in these cells is slower than in stimulated peripheral blood lymphocytes and H9 cells, presumably due to the low levels of deoxynucleotide triphosphate substrates in the nondividing cells (Collin and Gordon, 1994; O'Brien *et al.*, 1994).

Interestingly, as seen in Fig. 2A, more PCR product is obtained from ΔDU- than from WT-infected macrophages at all time points, indicating the presence of more viral DNA in the ΔDU-infected cultures. This may be a consequence of the ability of ΔDU EIAV to incorporate both dUTP and dTTP into viral DNA: perhaps reverse transcription can proceed more rapidly with the use of both deoxynucleotides in an environment where nucleotide pools might be low. Both *in vitro* and *in vivo* data indicate that uracil can be incorporated into viral DNA by EIAV RT. One critical function of cellular dUTPases is the maintenance of a low ratio of dUTP to dTTP in dividing cells, thereby indirectly preventing the incorporation of uracil into nascent DNA by DNA polymerases. RT assays indicate that EIAV RT can utilize either dUTP or dTTP for DNA synthesis (Figs. 3A and 3B). Studies in which recombinant dUTPase protein was added *in trans* to ΔDU virions or recombinant RT (Fig. 3C) suggest that the presence of dUTPase keeps local concentrations of dUTP low and prevents its incorporation into viral DNA, similar to the situation found with cellular dUTPases and DNA polymerases. The ability to add exogenous recombinant dUTPase to dUTPase-deficient virions, thereby restoring the "WT" phenotype, also suggests that an intimate association between dUTPase and other viral proteins (i.e., RT) is not required, although our results have not yet been confirmed by addition of heterologous (i.e., *E. coli*) dUTPase *in trans*.

Examination of ΔDU or DU^{D71E} viral DNA from macrophages by treatment of the DNA with uracil–DNA glycosylase followed by PCR amplification indicates this DNA does indeed contain uracil (Fig. 4). The most plausible explanation for higher levels of uracil in viral DNA in macrophages than in FEA cells is that macrophages, as nondividing cells, may have lower levels of dUTPase activity (and a higher dUTP:dTTP ratio) than the proliferating FEA cells.

As shown in Fig. 2B, some ΔDU and DU^{D71E} DNA becomes integrated in the macrophage, as evidenced by the presence of a EIAV-specific signal in the region of the blots containing high-molecular-weight DNA. Quantitation by densitometry indicates a two- to threefold decrease in the ratio of integrated to unintegrated in infections with the dUTPase-deficient viruses. Quantitation was performed using the amount of unintegrated viral DNA present in each lane to control for variables such as differences in input virus or efficiency of reverse transcription, as well as for any differences in sample

loading. In contrast to macrophages, both WT and ΔDU viruses integrated to an equivalent level in persistently infected FEA cells, indicating that the dUTPase region deletion did not influence viral integrase expression, processing, or function.

We are currently unable to determine if the decrease in the amount of integrated viral DNA in macrophages contributes significantly to the phenotype of decreased virus production. The estimate of a two- to threefold reduction in integrated DNA assumes that the hybridization signal is proportional to the number of full-length integrated proviruses. If the presence of uracil in viral DNA does result in deletions, recombination events, or an accumulation of point mutations during or after integration, then the amount of proviral DNA capable of supporting virus replication could be lower than estimated.

Transcription

The steady-state levels of viral RNA were examined by Northern blot analysis. As shown in Fig. 5, the steady-state level of ΔDU transcripts is decreased compared to that of the WT virus in both cell types. In FEA cells, we see a decrease of only about 3-fold in all viral transcripts and the proportions of multiply spliced, singly spliced, and unspliced mRNAs are similar for both viruses. In contrast, steady-state mRNA levels are much more dramatically decreased in ΔDU- and DU^{D71E}-infected macrophages. Quantitation of the Northern blot shown in Fig. 5A reveals about a 25-fold decrease in the level of multiply spliced transcripts, an 85-fold decrease in singly spliced transcripts, and a 300-fold decrease in the full-length message. The greater decrease in levels of singly spliced and full-length messages may reflect decreased levels of Rev protein encoded from multiply spliced transcripts. Other Northern blots with RNA recovered from infected macrophages were performed (data not shown) in which no ΔDU or DU^{D71E} viral transcripts were detected; therefore, the numbers stated above are minimal estimates. These experiments suggest that one of the blocks to replication of dUTPase-defective EIAV in macrophages is a severe decrease in the steady-state levels of viral transcripts.

Role of dUTPase in EIAV replication

Our findings indicate that the major block(s) to replication of dUTPase-deficient EIAV in macrophages occurs after synthesis of viral DNA and results in reduced levels of viral transcripts. The data also suggest that reduced replication results from the accumulation of uracil in viral DNA; however, we have not determined the molecular basis for these events.

One possibility is that viral integrase function might be affected by uracil-substituted DNA, although retroviral integrases recognize and require only 6 to 15 nucleotides at the ends of the linear viral DNA template (Bushman and Craigie, 1991; Katzman *et al.*, 1989; Sherman *et al.*, 1992; Vink *et al.*, 1991). Integration of intact proviruses might also be affected, however, if UDG and apyrimidinic endonuclease are present and active in adherent macrophages. Repair processes could lead to single-stranded regions resulting in increased levels of recombination, rearrangements, and deletions or an accumulation of point mutations. The loss of provirus integrity would affect all later steps in virus replication. Although levels of UDG are generally high during DNA replication and low in nondividing cells (Duker and Grant, 1980; Richards *et al.*, 1986), we cannot rule out that maintenance levels of these enzymes may be present in nondividing macrophages.

A second possibility is that transcription is decreased due to the stable incorporation of uracil in intact proviral DNA. The results of a single experiment in which UDG treatment and PCR amplification of integrated DNA were performed (data not shown) suggest uracil is present in integrated DNA from ΔDU-infected macrophages. It has been demonstrated in yeast that in a repair-deficient genetic background, the accumulation of uracil in DNA leads to cell death, apparently due to a generalized failure in macromolecular biosynthesis (Gadsden *et al.*, 1993); it was not determined if this was due to decreased or disregulated transcription. It has been demonstrated, however, that uracil, when substituted for thymine, can alter protein–DNA interactions *in vitro* in a variety of systems, including Fos–Jun complexes on the TPA responsive element (Risse *et al.*, 1989), HeLa cell nuclear proteins to the cAMP responsive element (Verri *et al.*, 1990), herpes origin-binding protein to its origin of replication (site I of Ori_S) (Focher *et al.*, 1992), *E. coli* RNA polymerase on the lambda P_R promoter (Dubendorff *et al.*, 1987), and the Lac repressor to the *lac* operator (Goeddel *et al.*, 1977).

In summary, we have demonstrated that EIAV requires its virally encoded dUTPase for efficient replication in macrophages, the primary host cell for this virus. It appears that reduced replication in macrophages results from the incorporation of uracil into viral DNA. We are currently attempting to quantitate uracil incorporation and are investigating the molecular events leading to reduced levels of viral transcripts. These studies may lead to a better understanding of the viral adaptations required for replication in nondividing host cells and may provide an *in vivo* model system for examining the effects of uracil in DNA.

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