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A Poxvirus-Encoded Uracil DNA Glycosylase Is Essential for Virus Viability

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Infection of cultured mammalian cells with the *Leporipoxvirus* Shope fibroma virus (SFV) causes the induction of a novel uracil DNA glycosylase activity in the cytoplasms of the infected cells. The induction of this activity, early in infection, correlates with the early expression of the SFV *Bam*HI D6R open reading frame which possesses significant protein sequence similarity to eukaryotic and prokaryotic uracil DNA glycosylases. The SFV *Bam*HI D6R open reading frame and the homologous *Hin*dIII D4R open reading frame from the *Orthopoxvirus* vaccinia virus were cloned under the regulation of a phage T7 promoter and expressed in *Escherichia coli* as insoluble high-molecular-weight aggregates. During electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, the *E. coli*-expressed proteins migrate with an apparent molecular mass of 25 kDa. The insoluble protein aggregate generated by expression in *E. coli* was solubilized in urea and, following a subsequent refolding step, displayed the ability to excise uracil residues from double-stranded plasmid DNA substrates, with the subsequent formation of apyrimidinic sites. The viral enzyme, like all other characterized uracil DNA glycosylases, is active in the presence of high concentrations of EDTA, is substrate inhibited by uracil, and does not display any endonuclease activity. Attempts to inactivate the *Hin*dIII D4R gene of vaccinia virus by targeted insertion of a dominant xanthine-guanine phosphoribosyltransferase selection marker or direct insertion of a frame-shifted oligonucleotide were uniformly unsuccessful demonstrating that, unlike the uracil DNA glycosylase described for herpesviruses, the poxvirus enzyme is essential for virus viability.

Most organisms safeguard their genetic material with several specific mechanisms for the repair of damaged or incorrectly incorporated bases (16). DNA glycosylases act at the initial stage of the pathway for excision repair of damaged bases by catalyzing the hydrolysis of the damaged base at the N-glycosylic bond (7). Uracil DNA glycosylases remove uracil residues that have been introduced into DNA either through misincorporation of dUTP by DNA polymerase or through the deamination of cytosine (26). The former event, leading to an A · U base pairing, is not necessarily mutagenic and occurs relatively infrequently owing to the low cellular pools of dUTP (17, 39). However, the latter event causes a G · U pairing that can result in a G · C to A · T base pair substitution if not repaired prior to DNA replication.

A family of DNA glycosylases with different substrate specificities allows cells to convert many different kinds of damage into a single lesion, an apurinic-apyrimidinic (AP) site. The AP site can be cleaved by AP endonucleases and then is repaired by a combination of DNA polymerase and DNA ligase activities (16). The significance of a pathway for uracil removal is apparent from the ubiquitous distribution of uracil DNA glycosylases in nature. To date, all prokaryotic and eukaryotic organisms screened have been found to express at least one form of uracil DNA glycosylase, and most eukaryotic organisms have both a nuclear form and an organelle form of the enzyme (5, 16, 18, 37). Furthermore, some insects have been shown to display developmentally regulated expression of the activity (6).

Until recently, mammalian herpesviruses were the only eukaryotic viruses known to encode a uracil DNA glycosylase (49). Infection with herpes simplex virus type 1 or type 2 results in a rapid induction of both dUTPase and uracil DNA glycosylase activities (2). The induction of these activities may be necessary in order to maintain the integrity of the viral DNA during a period of rapid DNA synthesis in the presence of greatly expanded cellular pools of DNA precursors (21). We have recently reported the identification of a gene encoding a functional uracil DNA glycosylase activity (SUG) within the Shope fibroma virus (SFV) genome (46). SFV is a member of the *Leporipoxvirus* genus (28) and is related to the prototypic *Orthopoxvirus* vaccinia virus (VV). Poxviruses are large DNA viruses that replicate in the host cell cytoplasm independently of any host nuclear functions (20, 33). By virtue of their cytoplasmic site of DNA synthesis, poxviruses are able to overcome cellular regulatory functions but must encode all of their own replicative and transcriptional enzymes. Previous studies have reported a lack of mismatch repair activities in SFV-infected cell cytoplasms (15), and in fact, extrahelical bases near the viral DNA termini are a conserved characteristic of the poxvirus genome (4). It was, therefore, of interest to discover that poxviruses encode an enzyme that functions in the initial phase of a DNA base excision repair pathway.

In this article, we report some aspects of the expression of the poxvirus uracil DNA glycosylase, characterize a bacterially expressed recombinant form of the enzyme, and present evidence that, unlike the herpesviruses, the VV uracil DNA glycosylase (VUG) is essential for poxvirus replication.

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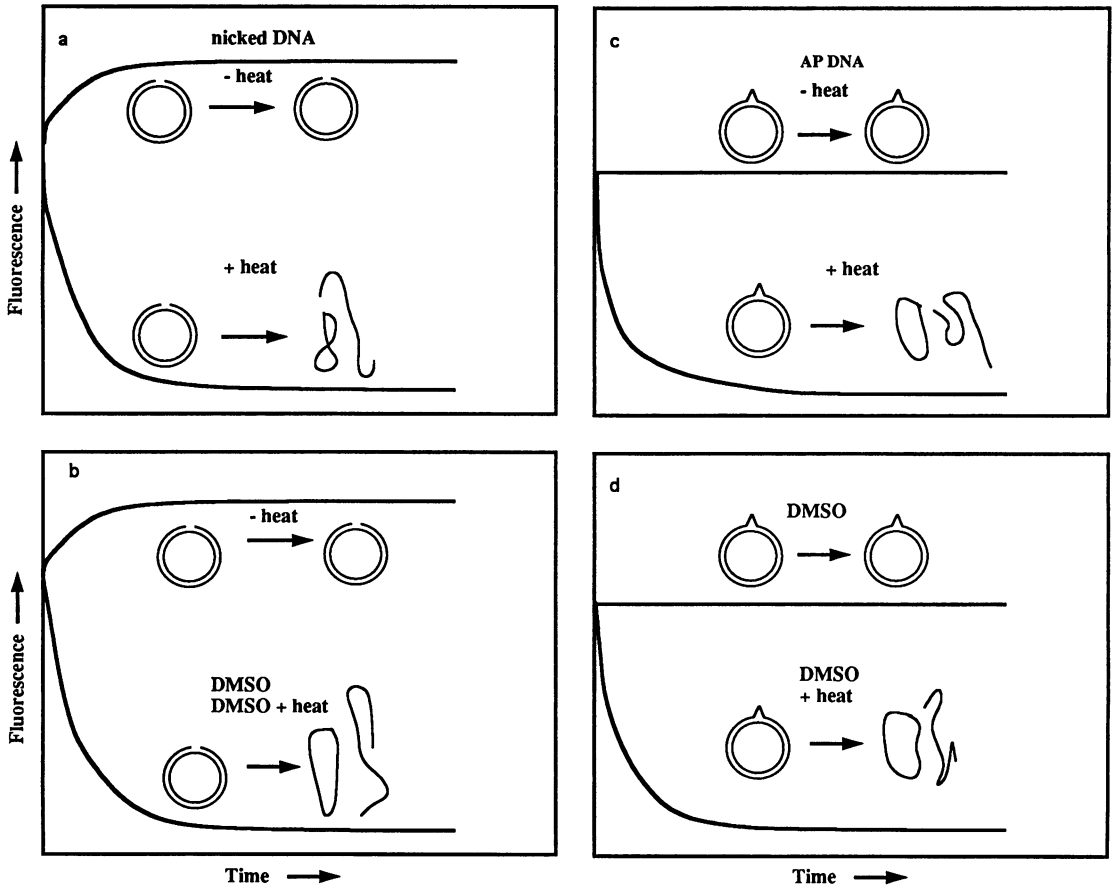

FIG. 1. Idealized graphs summarizing ethidium bromide fluorescence assays that detect the formation of nicks or AP sites in a circular DNA substrate. (a) The fluorescence of DNA as it becomes nicked by an endonuclease rises in the absence of heat, because OC DNA intercalates more ethidium bromide than CCC DNA; however, when the sample is heated to 95°C the DNA strands separate, with a subsequent loss of fluorescence. (b) The introduction of nicks into CCC DNA can be similarly detected by the loss of fluorescence following the denaturation of the sample with DMSO. (c) AP sites introduced into DNA by the action of a DNA glycosylase do not affect the level of intercalated ethidium bromide and, hence, the fluorescence of unheated DNA. Heating the AP DNA to 95°C hydrolyzes the AP sites, allowing the DNA strands to separate with a subsequent loss of fluorescence. (d) In the presence of DMSO, AP sites retain the sugar phosphate linkage, and thus, the ethidium bromide fluorescence of the DNA remains unchanged in contrast to the fluorescence of nicked DNA, which decreases as the strands separate as a result of denaturation. Heating AP site-containing DNA causes hydrolysis of the AP sites, leading to the loss of ethidium bromide fluorescence.

MATERIALS AND METHODS

Fluorescence assay for uracil DNA glycosylase. The ethidium bromide fluorescence assay to detect uracil DNA glycosylase activity has been described previously (12, 31). This assay is dependent upon the enhanced fluorescence of ethidium bromide when it has intercalated into duplex DNA. At pH 12, long duplex DNAs are stabilized by the intercalation of ethidium bromide while shorter duplexes are denatured, with a subsequent loss of fluorescence. Following thermal denaturation at 95°C, the topologically linked strands of covalently closed circular (CCC) DNA rapidly reanneal and regain all of the original fluorescence whereas the strands of a nicked circular DNA molecule separate and are prevented from reannealing by the high-pH condition (see Fig. 1a). Thus, the loss of fluorescence that occurs following heating can be used to monitor the creation of nicks in DNA. Note that, even in the absence of heating, the nicking of CCC DNA to the open circular (OC) form results in a slight increase in fluorescence because the OC DNA intercalates additional ethidium bromide since the ability to rotate the DNA strands allows greater unwinding of the helix (Fig. 1a, upper line). The treatment of uracil-containing DNA with a uracil DNA glycosylase results in the production of AP sites where uracil residues have been excised, but since the phosphodiester backbone of the DNA containing AP sites remains intact, no alteration in fluorescence over the CCC substrate DNA is observed (Fig. 1c). However, since AP sites are sensitive to hydrolysis under alkaline conditions, heating the DNA to 95°C at pH 12 for 5 min results in strand breakage at AP sites, and the subsequent irreversible denaturation is observed as a loss of fluorescence at pH 12 (Fig. 1c). Standard assay reactions for uracil DNA glycosylase activity contained 50 mM Tris-HCl [pH 7.5], 20 mM EDTA, 100 μg of heat-denatured gelatin per ml, and 1 μg of substrate DNA in a 50-μl volume. Additionally, 10-μl samples were divided into two aliquots, and one half of the sample was added directly to the pH 12 assay buffer to measure fluorescence while the other half was first mixed with 2 volumes of deionized dimethyl sulfoxide (DMSO). This treatment causes denaturation of nicked DNA but has

no effect upon the fluorescence of DNA that contains AP sites (12). By heating the DNA samples to 95°C and measuring the fluorescence and then comparing it with the fluorescence change caused by DMSO treatment alone, it was possible to determine whether AP sites had been generated and distinguish these from nicking of the DNA (Fig. 1b and d).

Substrate DNA, CCC plasmid DNA containing uracil, was prepared from *Escherichia coli* CJ236 containing pUC19 by isopycnic banding on CsCl gradients. CJ236 lacks both dUTPase (*dut*) and uracil DNA glycosylase (*ung*) activities, and thus, plasmid DNA isolated from this strain has uracil incorporated in place of thymine at a high frequency (22).

Cells and viruses. Cells used, BSC40, BGMK (both are African green monkey cell lines), and SIRC (a rabbit corneal cell line), were obtained from the American Type Culture Collection and were propagated as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (GIBCO Laboratories). SFV (strain Kasza) and VV (strain WR) were obtained from the American Type Culture Collection.

The induction of virus-specific uracil DNA glycosylase activity in virus-infected cells was determined by assaying crude cytoplasmic extracts made from monolayers of 10^6 SIRC cells that had either been mock infected or infected with SFV at a multiplicity of 10 infectious units per cell. The monolayers were harvested, after washing with cold phosphate-buffered saline (PBS) plus 5 mM EDTA, by scraping into 300 μl of cold PBS. The harvested cells were pelleted at 12,000 rpm in a Microfuge and resuspended in 100 μl of 10 mM Tris-HCl [pH 8.0]–10 mM EDTA–0.2% Nonidet P-40. The cells were incubated on ice for 15 min and then lysed by multiple passages through a small-bore pipette tip, the nuclei and cell debris were pelleted by low-speed centrifugation, and the supernatants were snap frozen in a dry ice-ethanol bath and stored at −70°C. Five micrograms of the total protein from each lysate was used in the fluorometric assay for uracil DNA glycosylase activity.

Cloning and expression of the SFV *Bam*HI D6R and VV *Hin*dIII D4R genes. The cloning of the SFV *Bam*HI D6R open reading frame (ORF) (SUG) into vector pET3a is described elsewhere (46). In this vector, the SUG gene is placed under the transcriptional control of the phage T7 RNA polymerase. In a similar manner, the VUG gene was also cloned into pET3a after polymerase chain reaction amplification of the VV *Hin*dIII D4R ORF from VV genomic DNA (11). The 5′ primer incorporated a unique *Nde*I restriction site (5′-GGCATATGAATTCAGTGACTGTATC-3′), and the 3′ primer incorporated a unique *Bam*HI site (5′-GGGGATCCTAAAATTTCACTAAAGC-3′). The VV *Hin*dIII D4R ORF was amplified by 20 cycles at 96°C for 30 s, 55°C for 45 s, and 72°C for 45 s. The amplified 660-bp fragment was purified by preparative gel electrophoresis (23). The expression vectors thus derived were named pXSUG-1 (SFV *Bam*HI D6R gene) and pXVUG-1 (VV *Hin*dIII D4R gene). The VUG gene was also cloned into pET19b (Novagen) to create p19VUG-1, a derivative of pET3a which places a 23-amino-acid peptide (M-G-H-H-H-H-H-H-H-H-H-S-S-G-H-I-D-D-D-D-K-H-) at the NH_2 terminus of the expressed protein and facilitates purification by affinity chromatography via binding to divalent cations (Ni^{2+}).

Two *E. coli* strains were used for the expression of the SUG gene. The plasmids described above were transformed into *E. coli* CJ236 (22), which lacks dUTPase and uracil DNA glycosylase activities (*dut ung*), and *E. coli* BL21(DE3) (43), which is protease deficient and carries the phage T7 RNA polymerase under the control of an inducible *lac* UV promoter. Induction of T7 RNA polymerase activity with the subsequent expression of genes under the regulation of a T7 promoter can be achieved by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the growth medium of cultures in mid-log phase. Three hours postinduction, the cells were harvested by centrifugation, and the cell pellet was stored at −70°C. Upon thawing, the cells were lysed (by the addition of 50 mM Tris-HCl [pH 7.5]–150 mM NaCl–10 mM EDTA–1 mM dithiothreitol–10% sucrose–0.2 mg of lysozyme per ml) and incubated on ice for 15 min, and then Triton X-100 was added to 0.1% and the cell debris was pelleted by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor at 4°C for 20 min. An induced 25-kDa protein was found as an insoluble aggregate in the pellet. This insoluble protein pellet was washed three times with lysis buffer plus 0.5% Triton X-100, collected by centrifugation at 12,000 rpm in a Microfuge, solubilized in 8 M urea, and allowed to incubate for 2 h at room temperature. The remaining insoluble material was removed from the solution by centrifugation at 12,000 × *g* for 10 min in a Sorvall SS-34 rotor. The supernatant was then incubated at 4°C for 2 h, and the concentration of urea was diluted to 4 M by the slow addition of 50 mM Tris-HCl [pH 7.5]–10 mM EDTA–50 mM KCl–0.01% Nonidet P-40 over 3 h, after which the solution was dialyzed for 16 h against 100 volumes of the dilution buffer with four changes. The refolded protein was tested for uracil DNA glycosylase activity in the fluorescence assay and then concentrated to 1 ml by ultrafiltration using a Centricon 10 concentrator (Amicon) at 4°C. The refolded monomer size protein was then separated from the reaggregated fraction and some of the remaining *E. coli* proteins by gel filtration chromatography over a 15-ml column of Sephacryl S-300 (Pharmacia), 500-μl fractions were collected and tested for activity, and the active fractions were pooled, concentrated by ultrafiltration, and stored in 50% glycerol at −20°C.

Northern blot analysis. RNA for Northern (RNA) blots was prepared by infection of monolayers of 3.5×10^6 BGMK cells in 100-mm dishes with SFV at a multiplicity of 10 infectious units per cell. The infections were allowed to proceed for 16 h to isolate late RNA or were allowed to proceed for 16 h in the presence of 100 μg of cycloheximide per ml to collect RNA from genes expressed exclusively prior to DNA replication (early RNA). The cells were lysed in situ by the addition of 2.5 ml of 6 M guanidinium isothiocyanate–0.5% Sarkosyl–0.1 M β-mercaptoethanol–5 mM sodium citrate [pH 7.0]. The cell lysate was scraped from the plate, and DNA was sheared by five passages through a 26-gauge needle. Solid $CsCl_2$ (1.0 g) was dissolved in each 2.5 ml of homogenate, this solution was layered onto a 2.5-ml cushion of 5.6 M $CsCl_2$–100 mM EDTA, and the RNA was pelleted by centrifugation at 37,000 rpm in an SW50.1 rotor for 17 h at 20°C. The RNA was resuspended in diethyl pyrocarbonate-treated water, extracted three times with chloroform, and then precipitated with ethanol. Ten micrograms of the early or late total RNA was loaded into each lane of a 1% agarose–2.2 M formaldehyde gel and fractionated by electrophoresis (38). Following capillary transfer to a Hybond-C membrane (Amersham) and UV cross-linking, the RNA was hybridized with an SFV D6R-specific probe that had been internally labeled with [α-^{32}P]dATP by random priming with extension of the primers by the Klenow fragment of DNA polymerase I (14). Following stringent washing, the blots were visualized by autoradiography.

Mutagenesis of the VUG gene. A 2,819-bp *Bam*HI-*Bgl*II fragment containing the complete VV *Hin*dIII D4R (VUG) and the D3R ORFs (35) was isolated and inserted into the *Bam*HI site of pUC19 (see Fig. 7). This plasmid construct, referred to as p813a, was used as the target for in vitro mutagenesis.

Two mutagenesis procedures were applied in an attempt to create a null allele of the VUG gene in the VV genome. A frameshift mutation was introduced into the D4R ORF by insertion of an 8-bp oligonucleotide linker into the unique *Eco*RV site of p813a (p813aBglII). Sequencing analysis revealed that 1 bp of the VUG coding sequence was lost, thus creating a truncated gene consisting of the normal N-terminal 179 amino acids plus an additional 2 amino acids derived from the plasmid sequences but lacking the C-terminal 40 amino acid residues. A second vector was designed to target the dominant selectable *E. coli* xanthine-guanine phosphoribosyltransferase (*gpt*) gene into the VUG gene. Plasmid p813agpt was generated by inserting a *Dra*I fragment, containing the *E. coli gpt* gene downstream of VV transcriptional regulatory sequences derived from pTKfs3 (13), into the *Eco*RV site of p813a. Insertion of this marker by homologous recombination into the genomic VUG locus would confer mycophenolic acid (MPA) resistance to progeny virus.

The procedures used to generate recombinant viruses have been described in detail elsewhere (13, 35, 36). Briefly, the first approach involved the infection of BSC40 cells with VV strain *ts*C35, which possesses a temperature-sensitive lesion in the *Hin*dIII D3R ORF (10). Following infection, the cells were transfected with the mutagenesis vector p813aBglII. This vector contains a copy of the wild-type D3R gene, and viruses that have incorporated the wild-type sequence can be selected by growth at 40°C, a temperature which is nonpermissive for the C35 allele. All virus plaques that were isolated from this primary screen were propagated at 37°C. In each case, the structure of the VUG gene was determined initially by dot blot (48) and then by Southern blot analysis. The dot blots were probed either with a 5′-end-labeled oligonucleotide that conformed to the wild-type VUG gene sequence (5′-ACCATAGTCGGATATC ATCCAGCGG-3′) or with an oligonucleotide matching the sequence of the frame-shifted version of the VUG gene (5′-TAGTCGGACAGATCTGATCATCC-3′). The Southern blot probe corresponded to the 1,034-bp *Eco*RI-*Bgl*II fragment containing the D4R gene, labeled by nick translation.

In the second experiment, BSC40 cells were infected with VV (strain WR) at a multiplicity of 0.2 PFU per cell. Two hours postinfection, the infected monolayers were transfected with p813agpt. Following a 4-h incubation, the tissue culture medium was aspirated from the monolayers and replaced with fresh medium; at 48 h postinfection, the monolayers were harvested by scraping, and virus stocks were prepared. The virus obtained from this infection was then used to select for MPA-resistant virus. Confluent monolayers of BSC40 cells were preincubated in selection medium (Dulbecco's modified Eagle's medium, 2.5% fetal bovine serum, 25 μg of MPA per ml, 250 μg of xanthine per ml, 15 μg of hypoxanthine per ml) for 14 h and infected with serial 10-fold dilutions of the stock virus. After 2 h, the monolayers were overlaid with a *gpt* selective medium containing 1% low-melting-point agarose, incubated for 2 days, and stained with neutral red (GIBCO Laboratories), and then virus plaques were isolated. All of the MPA-resistant plaques that were obtained by this procedure were propagated in the presence of MPA, and the genome structure in the region of the VUG gene was determined by Southern blot analysis using either a VUG gene-specific fragment or sequences from the *gpt* cassette.

RESULTS

Induction of uracil DNA glycosylase activity in SFV-infected cells. Infection of mammalian cells with poxviruses results in the induction of a series of enzymatic activities that are involved in nucleic acid metabolism. Elsewhere, we report the first demonstration of a poxvirus DNA repair enzyme, a uracil DNA glycosylase encoded by SFV (SUG) and also note the presence of homologous genes in the data bases of VV and fowlpox DNA sequences (46). Here, we have used the ethidium bromide fluorometric technique to further characterize this enzyme encoded by SFV and VV. As described in Materials and Methods, and in the legend to Fig. 1, the assay relies on the ability of intact supercoiled CCC plasmid containing uracil residues to renature following DMSO or heat denaturation at pH 12 and thus regain the level of ethidium bromide fluorescence detected before denaturation. Nonspecific nicking of the CCC substrate can be distinguished from uracil DNA glycosylase activity, because the former causes fluorescence loss by either DMSO treatment or heating at pH 12 while the latter produces AP sites which retain the sugar phosphate linkage and thus are unaffected by DMSO. A further control is the inability to detect this activity with CCC DNA substrate lacking uracil. Thus, the ability to induce ethidium bromide fluorescence loss in uracil-containing CCC DNA by DMSO-and-heat treatment but not by DMSO treatment alone is a specific characteristic of uracil DNA glycosylase. Initially, crude lysates of SFV-infected cells were assayed for the presence of this virally induced uracil DNA glycosylase. Cytoplasmic extracts from cells that had been mock infected or infected with SFV for various periods of time were assayed. An induction of SUG activity above the background level of host uracil DNA glycosylase activity was detected as early as 2 h postinfection, and this level of activity was retained over the first 6 h of the infection before declining in the late stages of the infection (Fig. 2a). The decrease in fluorescence shown in Fig. 2a is indicative of the introduction of heat-induced nicks into the uracil-containing substrate DNA at AP sites and its denaturation. The fluorescence of an identical DNA substrate that lacked any incorporated uracil remained unchanged over the time course of the experiment following treatment with the 2-h lysate (Fig. 2b), indicating that the alteration in fluorescence of the uracil-containing substrate is due to the specific activity of a uracil DNA glycosylase that is induced prior to the initiation of viral DNA replication. The constant decrease in fluorescence seen in the values obtained after heating reflects less than 100% reannealing of the substrate DNA (Fig. 2b), but this value is consistently observed for all samples tested. Thus, a significantly elevated level of uracil DNA glycosylase activity is observed upon infection of SIRC cells with SFV. When comparable infections were carried out in the presence of cycloheximide to inhibit induced viral protein synthesis, no induction of uracil DNA glycosylase was detected (data not shown), suggesting that the observed activity was the result of de novo viral gene expression rather than a component of the infecting virions.

SUG has previously been shown to be encoded by the *Bam*HI D6R ORF of SFV (46). Oligonucleotide primers from the 5′ and 3′ ends of this ORF were used to amplify a DNA fragment containing the complete SUG coding sequence,

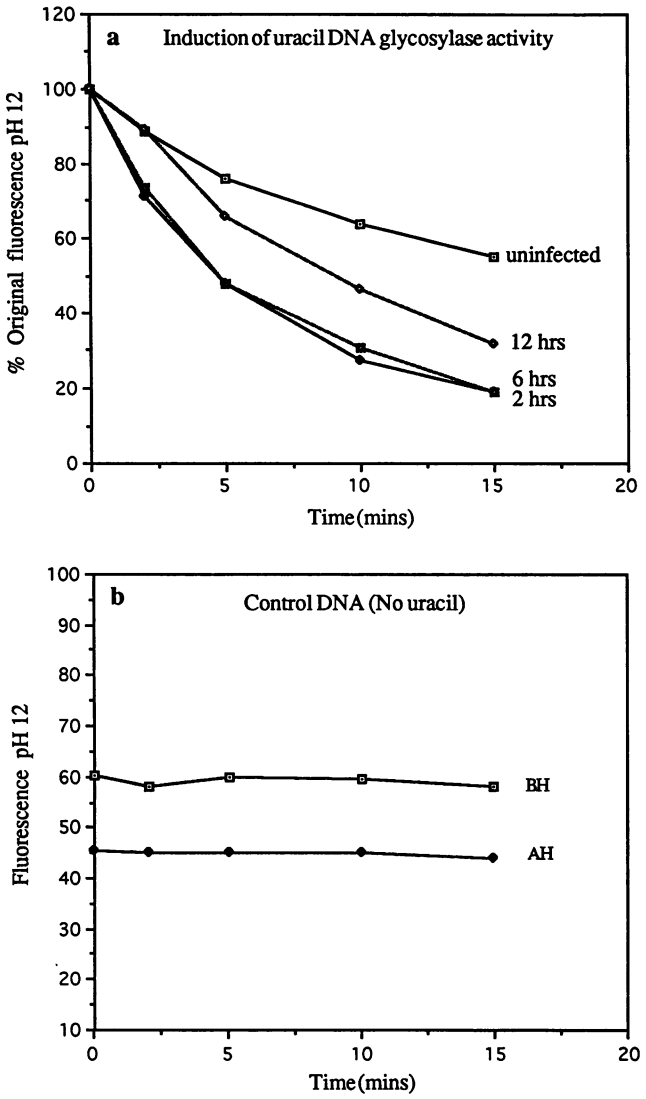

FIG. 2. Ethidium bromide fluorescence assays detect the induction of a uracil DNA glycosylase activity in the cytoplasms of SFV-infected cells. All of the reactions were done at 37°C in the presence of 20 mM EDTA to minimize the influence of nucleases. (a) Fluorescence profiles after heat denaturation of uracil-containing plasmid DNA substrates after incubation with cytoplasmic extracts made from cells that had been either infected with SFV for 2, 6, or 12 h or mock infected. The loss of fluorescence during the assay is indicative of the formation of AP sites or nicks in the test plasmid DNA. (b) Fluorescence profiles of a control DNA substrate without incorporated uracil before heating (BH) and after heating (AH) following incubation with a cytoplasmic extract made from cells that had been infected with SFV for 2 h. The stable fluorescence during the assay suggests that no random nicking has occurred.

which was then labeled with [α-^{32}P]dATP by the random priming technique and used as a hybridization probe for Northern blots of total RNA isolated from SFV infected cells. The SUG probe hybridizes most strongly to a transcript of approximately 700 nucleotides and also to a minor product of higher molecular weight (Fig. 3, lane 1). The major transcript is detected in RNA prepared from cells infected with SFV in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3, lane 1), a condition which

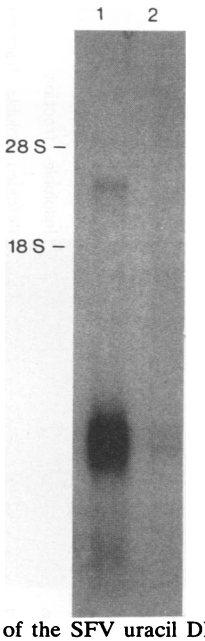

FIG. 3. Expression of the SFV uracil DNA glycosylase (D6R) gene. A Northern blot of total RNA isolated from SFV-infected cells and probed with a ^{32}P-labeled SUG ORF is shown. Lane 1, early RNA; lane 2, late RNA. The positions of cellular 28- and 18S rRNAs are indicated on the left.

prevents viral DNA replication and the expression of intermediate and late genes. Some of the SUG transcript is still detected in RNA prepared at late stages (16 h) of the infection (Fig. 3, lane 2), suggesting that it is a relatively stable message. Although formal characterization of the SFV SUG promoter remains to be done, the accumulation of SUG mRNA in the absence of DNA replication indicates that the gene is expressed at early times. Similarly, the homologous VV *Hin*dIII D4R ORF is expressed as an early gene at the 5′ end of a large transcript that has both D4R and D5R coding sequences, of which only D4R is translated into protein (25). These results are consistent with the requirement for the poxvirus DNA repair enzyme during replication of the viral genome.

Characterization of recombinant SUG. Plasmid pXSUG-1 is a pET3a derivative in which SUG is placed under the control of the phage T7 transcriptional system (46). In an attempt to express SUG in a host cell with a *ung* mutation background, pXSUG-1 was transformed into the *E. coli* CJ236 *dut ung* mutant. Although pXSUG-1 was stable in this strain while no source of T7 RNA polymerase was present, when we tried to introduce T7 RNA polymerase into these cells by transformation with plasmid pGP2-1, which carries the phage T7 RNA polymerase gene under control of the *lac* UV promoter and a marker for kanamycin resistance (44), we were unable to isolate clones that contained both plasmids (i.e., ampicillin- and kanamycin-resistant clones). We presume that even low levels of expression of SUG in the *dut ung* mutant strain are lethal since (i) cotransformants could be obtained with pGP2-1 and the parental vector pET3a and (ii) it was also possible to transform the *dut*$^+$ *ung*$^+$ strain HB101 with pGP2-1 and pXSUG-1. It is likely that the action of SUG on the abnormally high number of uracil residues in the chromosome of *E. coli* CJ236 results in the loss of genome integrity and death of the host cell.

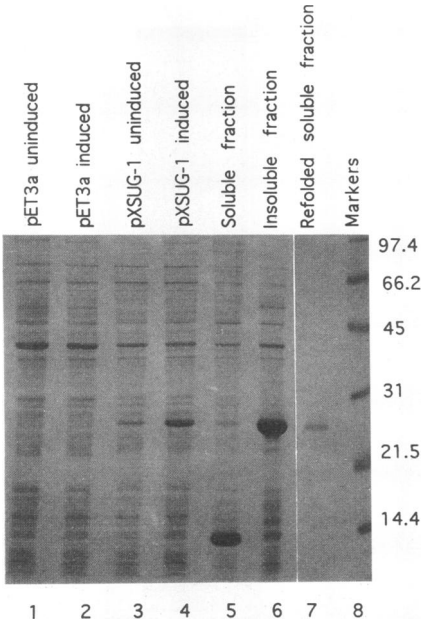

FIG. 4. Expression of the SUG gene in *E. coli* BL21(DE3). Conditions for cell growth and induction of SUG by IPTG are described in the text. Cell lysates harvested 3 h postinduction were fractionated on a sodium dodecyl sulfate–12% polyacrylamide gel, and the protein profiles were visualized by staining with Coomassie blue. Lane 1, total-cell lysate from BL21(DE3) harboring the parent vector pET3a prior to induction with IPTG; lane 2, total-cell lysate from BL21(DE3) harboring pET3a made 3 h after the addition of 0.4 mM IPTG to the growth medium; lane 3, total-cell lysate from BL21(DE3) harboring pXSUG-1 prior to induction with IPTG; lane 4, total-cell lysate from BL21(DE3) harboring pXSUG-1 made 3 h postinduction with IPTG; lane 5, the soluble protein fraction following a 12,000 × *g* centrifugation of a cell lysate made from BL21(DE3) carrying pXSUG-1 3 h postinduction; lane 6, the insoluble protein fraction of the lysate shown in lane 5; Lane 7, the soluble protein fraction following refolding and S-300 gel filtration chromatography. Molecular sizes in kilodaltons are shown on the right.

Since a demonstration of SUG activity by a direct complementation strategy was not possible, pXSUG-1 was used for expression studies with *E. coli* BL21(DE3). This strain contains a lambda prophage with T7 RNA polymerase under the control of a *lac* UV promoter which can be effectively induced by IPTG. Under such induction conditions, accumulation of a 25-kDa protein in the total-cell lysates of *E. coli* BL21(DE3)pXSUG-1 was observed (Fig. 4, lane 4) but not prior to induction. The 25-kDa band is in good agreement with the calculated SUG product of the 217-amino-acid ORF. The induced protein accumulated in insoluble inclusion bodies that were quantitatively removed from the lysate by low-speed centrifugation (Fig. 4, lane 6). *E. coli* BL21(DE3) is wild type for uracil DNA glycosylase, and since the vast majority of SUG protein was produced as insoluble material, it was not possible to detect an induction of activity over the host background activity in the total-cell lysates by using the fluorometric assay (not shown). However, when the isolated inclusion bodies were solubilized in 8 M urea, extensively dialyzed at 4°C, and then purified on an S-300 gel filtration column (Fig. 4, lane 7), a significant amount of uracil DNA glycosylase activity from the refolded

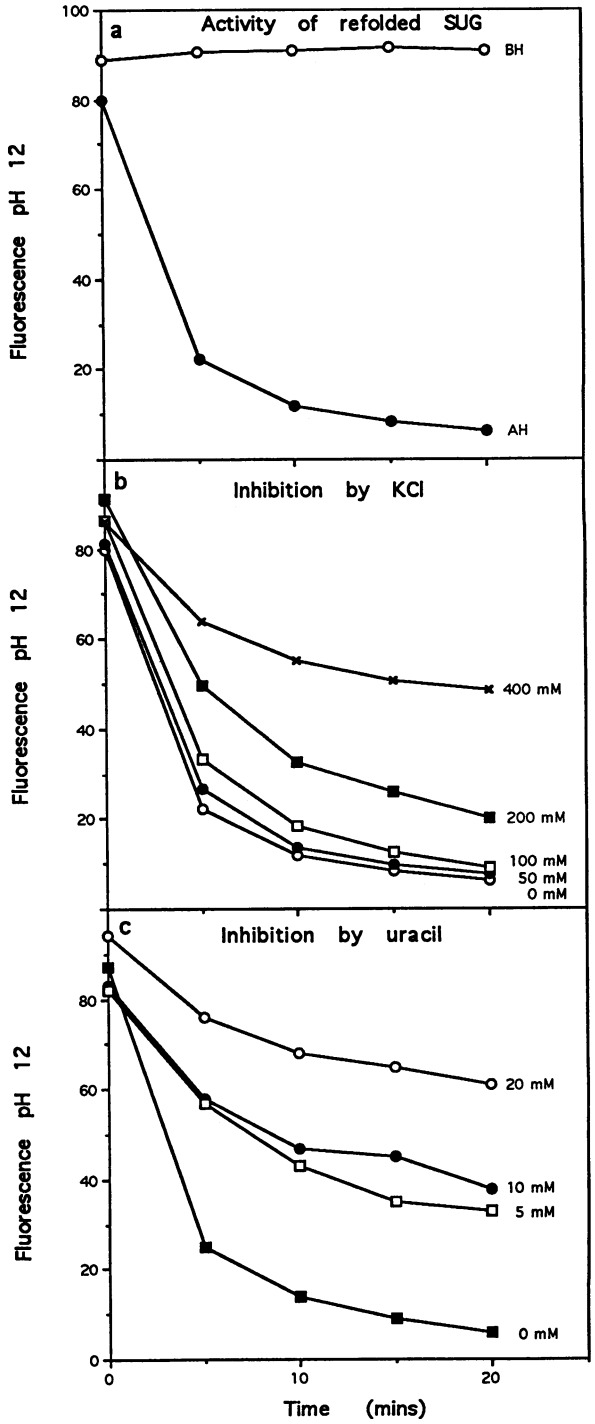

FIG. 5. SUG expressed in *E. coli* and isolated as inclusion bodies can be denatured and refolded into an active form. Fluorescence assays using a uracil-containing plasmid DNA substrate are shown. (a) Fluorescence of the substrate DNA following reaction with the enzyme before heating to 95°C (BH) and after heating to 95°C for 5 min (AH). (b) Activity of the SFV uracil DNA glycosylase as a function of KCl concentration. The fluorescence profiles of uracil-containing plasmid DNA after it has been reacted with the refolded enzyme in the presence of the indicated concentrations of KCl and heated to 95°C for 5 min are shown. (c) Product inhibition of the refolded SFV uracil DNA glycosylase by reactions in the presence of the indicated concentrations of uracil. The fluorescence profiles of uracil-containing plasmid DNA after reaction with the refolded enzyme and heating to 95°C for 5 min are shown.

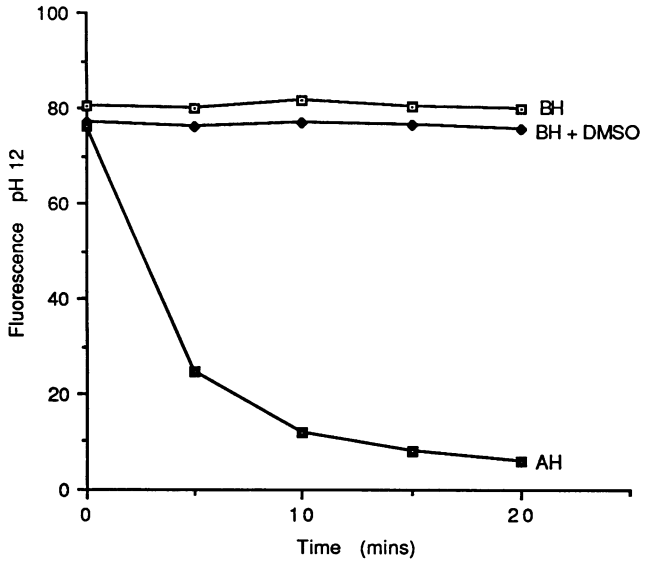

FIG. 6. Ethidium bromide fluorescence assay of the refolded SFV uracil DNA glycosylase using uracil-containing plasmid DNA as a substrate. Following treatment with the enzyme, the fluorescence of the plasmid DNA was measured at pH 12 before heating (BH), after the addition of DMSO to denature nicked DNA (BH + DMSO), and then in the presence of DMSO after heating to 95°C for 5 min (AH).

SUG polypeptide was detected (Fig. 5). Low levels of uracil DNA glycosylase activity in the insoluble fraction isolated from BL21(DE3) cells that did not carry the expression vectors were sometimes detected, but this activity was very low and could not be detected consistently.

The SUG has maximal activity under low-salt conditions. Full activity was observed with reaction mixtures that contained up to 50 mM KCl, but increasing the salt to 100 mM was detrimental to activity, and a drastic loss of activity was observed with 200 and 400 mM KCl (Fig. 5b). SUG was product inhibited, as shown by assays, in the presence of increasing concentrations of uracil, although complete inhibition did not occur even in the presence of 20 mM uracil (Fig. 5c).

Since no increases in fluorescence readings were detected during the SUG assays before the denaturation step (Fig. 6, BH), it is likely that SUG does not possess AP endonuclease activity, since either random nicks or breakage of the DNA backbone at AP sites would relax the CCC substrate to the OC form and, in turn, result in increased binding of ethidium bromide and fluorescence readings above that at the initial time point (12). This result was confirmed by performing a modified uracil DNA glycosylase assay in which the samples were mixed with 2 volumes of DMSO before addition to the pH 12 assay buffer. Nicked DNA is fully denatured under these conditions, while DNA with AP sites (CCC) remains intact. As shown in Fig. 6, substrate DNA showed no loss of fluorescence after reaction with the partially purified SUG plus treatment with DMSO, thus demonstrating the absence of phosphodiester strand cleavages. However, when the samples were heated to 95°C for 5 min, fluorescence was lost, indicating that SUG, like all other known uracil DNA glycosylases, excises the uracil base from DNA to create an AP site but does not induce incisions in the phosphodiester backbone at that site.

The VV uracil DNA glycosylase encoded by ORF *Hin*dIII D4R is an essential gene. VV ORF *Hin*dIII D4R encodes a polypeptide (VUG) that is 68% identical to SUG and represents an ORF which, on the basis of current knowledge, is the most highly conserved between the ortho- and leporipoxviruses (46). Uracil DNA glycosylase activity was demonstrated for the polypeptide encoded by this VV ORF by the same procedures as that for SUG. The VV *Hin*dIII D4R ORF was polymerase chain reaction amplified by using primers which incorporated *Nde*I and *Bam*HI sites into the 5′ and 3′ ends of the fragment, respectively, and cloned into the pET3a and pET19b expression vectors. In vitro transcription and translation from pXVUG-1 and refolding of inclusion bodies expressed from pXVUG-1 in *E. coli* BL21(DE3) both yielded uracil DNA glycosylase activities that were indistinguishable from that produced by pXSUG-1. In addition, assay of soluble protein expressed from p19VUG-1 in *E. coli* BL21(DE3) and purified by affinity chromatography (binding to a divalent cation column) similarly indicated that the VV *Hin*dIII D4R polypeptide is a functional uracil DNA glycosylase (data not shown).

In order to further characterize the function of the poxvirus uracil DNA glycosylase in vivo, we attempted to generate a mutant VV containing a disrupted VUG allele by two complementary genetic approaches. In the first, a plasmid containing both the VV *Hin*dIII D3R and the VV *Hin*dIII D4R genes was mutated by the incorporation of a *Bgl*II linker into the coding sequence of the D4R gene (VUG) to generate a frameshift and premature stop codon (Fig. 7). This plasmid (p813aBglII) was employed in a single-step marker rescue of the temperature-sensitive mutation in the D3R gene of VV strain *ts*C35. Temperature-insensitive progeny virus plaques were isolated, and the structure of the VUG gene was determined for each isolate by blot hybridization. The progeny generated in this experiment had either a single wild-type VUG gene or a mixture of both wild-type and mutant genes. The former isolates are generated by a double crossover event within the D3R gene sequences that rescues the temperature-sensitive mutation in D3R but fails to introduce the frame-shifted VUG allele into the viral genome. The latter group represents viruses that have incorporated the mutant gene but also retained a copy of the wild-type VUG. In order to confirm this observation, the 15 virus isolates that exhibited a mixture of the wild-type and mutant D4R genes were again used to infect BSC40 cells at a low multiplicity of infection, and single plaques were isolated at 40°C. DNA was prepared from these isolates, and the structure of the D4R gene was determined by Southern blotting. Of these 15 isolates, 10 possessed the wild-type VUG gene and 5 still retained a mixture of the wild-type and mutant alleles, indicating either that some of the original isolates were not pure or that the extra copy of the VUG gene had been lost. It is important to note that no virus progeny containing only the mutant VUG could be isolated. In contrast, when a similar approach was applied to VV gene D8L, a frameshift mutant was isolated from the first six plaques analyzed (36).

In a second attempt to disrupt the VUG gene, plasmid p813agpt (Fig. 7) was used to replace the wild-type VUG gene with a disrupted copy of the gene by homologous recombination. MPA-resistant virus was isolated, plaque purified, and propagated in the presence of MPA, DNA was prepared from 18 independent virus plaques, and Southern blot analysis was used to determine the structure of the viral genome in the VUG gene region. All 18 virus preparations possessed a mixture of the wild-type and disrupted VUG

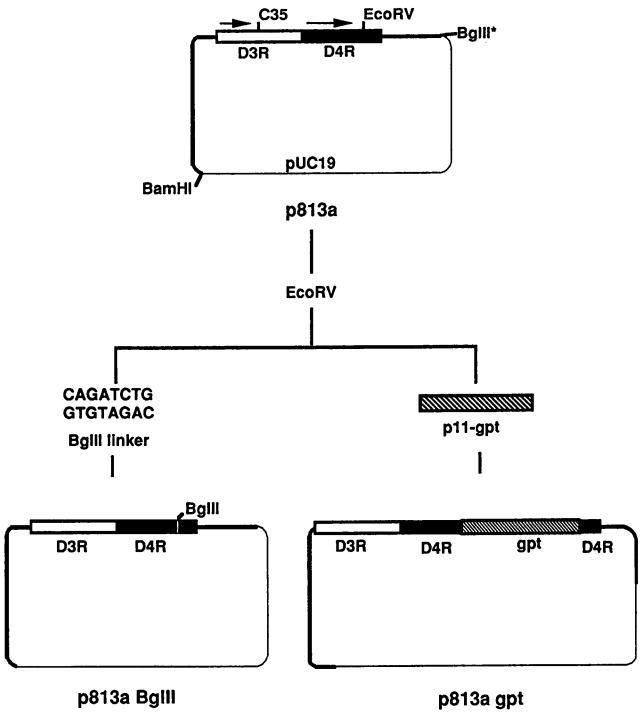

FIG. 7. Construction of vectors to disrupt the VV D4R gene by homologous recombination. Plasmid p813a contains a 2,819-nucleotide *Bam*HI-*Bgl*II fragment derived from the VV *Hin*dIII D region. The *Bgl*II site denoted by the asterisk was lost by virtue of the cloning procedure. This fragment, which extends from within gene D1R though a portion of gene D5R including the entire D3R and D4R coding sequence, was ligated into the *Bam*HI site of pUC19. Mutant versions of the D4R gene were generated by cleaving p813a with *Eco*RV, which cuts uniquely within the D4R coding sequence, and inserting either an 8-bp *Bgl*II oligonucleotide linker to generate p813aBglII or the *E. coli gpt* gene under the control of a VV promoter sequence to generate p813agpt. VV sequence is indicated by thick lines. The locations of the unique *Eco*RV site and the temperature-sensitive lesion in *ts*C35 are indicated.

genes. Again, no virus isolates possessed only the disrupted copy of the VUG gene. Since we were unable to isolate a virus that possessed only a mutant allele of the D4R gene in the absence of a wild-type allele, we conclude that expression of this gene is essential for virus propagation in tissue culture.

DISCUSSION

We have characterized a uracil DNA glycosylase encoded by the *Bam*HI D6R ORF of the *Leporipoxvirus* SFV and have found that the homologous gene (*Hin*dIII D4R ORF) from the *Orthopoxvirus* VV also encodes a functional uracil DNA glycosylase. Furthermore, our results indicate that the VV *Hin*dIII D4R gene is essential for the viability of VV, and we presume that the D6R gene is also critical for SFV. Poxviruses replicate in the host cell cytoplasm by virtue of an array of virally encoded DNA replicative enzymes (32, 45). Several factors may contribute to the requirement for a virally encoded uracil DNA glycosylase. Extensive poxvirus DNA synthesis occurs in an apparently unregulated fashion, supported by an expanded cellular pool of DNA precursors (40). Poxvirus infection specifically elevates the synthesis of DNA precursors by the expression of viral enzymes such as thymidine kinase (19), ribonucleotide reductase (41), and thymidylate kinase (42). High intracellular nucleotide concentrations may influence the probability of misincorporating dUTP in place of dTTP, and this might also explain the need for the putative poxvirus-encoded dUTPase (29). The rapid shutoff of most host cell functions following poxvirus infection may also limit the amounts of the host dUTPase and uracil DNA glycosylase activities available. It has been reported that the cellular levels of these enzymes are very low in quiescent cells (3), and thus, a viral homolog of each of these activities may be required to sustain high levels of accurate incorporation during DNA replication. Poxvirus replication produces considerable amounts of single-stranded DNA (45), which is more susceptible to hydrolytic deamination of cytosine residues than is duplex DNA (26); therefore, successful replication and minimization of mutation rates may require that poxviruses encode their own uracil DNA glycosylases along with other replicative enzymes.

Uracil DNA glycosylase activity is induced rapidly following poxvirus infection, suggesting that the enzyme is required prior to and during DNA synthesis rather than as a postreplicative repair activity. Similar conclusions have been drawn with respect to the nuclear form of the enzyme in mammalian cells, in which an increase in uracil DNA glycosylase activity slightly precedes the peak of DNA synthesis in normally cycling or stimulated cells (18, 47). The SUG gene contains two consensus signals for the termination of early viral transcription (TTTTTAT). The termination signal occurring at the 3′ end of the gene is consistent with the size of the 700-bp transcript, but the apparent termination signal that occurs just 12 nucleotides from the translation initiation site is likely not utilized. The mechanism that regulates this antitermination phenomenon is not understood, but it may involve the formation of secondary structure in the nascent RNA (27) and could function to downregulate the expression of this gene. VUG is also expressed early in the replicative cycle, although the transcript encoding the VV gene is not terminated at the 3′ end of the gene. Rather, a single transcript of 3.6 kb originates from the VUG promoter, and transcription reads through the downstream gene, terminating within the coding sequence of the next ORF (25).

We have attempted to inactivate the *Hin*dIII D4R VUG gene in VV using two distinct protocols. The introduction of a frameshift mutation and disruption of the VUG gene with a dominant selectable marker were both attempted, but for each and every clone tested only virus possessing a wild-type VUG allele or mixtures of wild-type and mutant alleles was isolated. We believe that this is strong evidence that the poxvirus uracil DNA glycosylase serves an essential function. This suggests that the removal of uracil incorporated into the viral genome may be particularly important for poxviruses, although several other explanations are possible. The uracil DNA glycosylase gene can be deleted from or disrupted in *E. coli* (8), *Saccharomyces cerevisiae* (1) and herpes simplex virus (34), indicating that the activity is not absolutely essential for growth and DNA replication in these organisms. However, both the *E. coli* and yeast mutants display increased sensitivities to mutagens (1, 8), and the *E. coli* mutant displays an elevated spontaneous mutation rate (9). To date, no reports of mutations in the VV uracil DNA glycosylase gene have been published. However, a conditional lethal mutation in VV strain IHD-W has recently been mapped to the carboxy-terminal portion of the D4R gene (3a), and this mutant virus, designated ts4149, displays a

marked defect in DNA synthesis at the nonpermissive temperature (28). The mapping of this conditionally lethal mutation within the VUG gene, along with our inability to generate a virus encoding a carboxy-terminal truncation of the gene, suggests that the carboxy-terminal region could contain elements of the catalytic domain or else be essential for interaction with other viral polypeptides.

The reason that this DNA repair enzyme is apparently essential for viral DNA synthesis is not clear. One hypothesis is that SUG and VUG have additional activities that remain to be elucidated. The ability of a polypeptide-encoding uracil DNA glycosylase activity to serve other apparently unrelated functions is not without precedent. The 37-kDa subunit of the human glyceraldehyde-3-phosphate dehydrogenase, which functions in the glycolytic pathway as a homotetramer, displays base excision activity in vitro in the monomeric configuration (30). Another explanation for the essential nature of this protein in DNA replication is based on the observation that a human uracil DNA glycosylase copurifies with DNA polymerase alpha (24). The poxvirus uracil DNA glycosylase may similarly reside within a multisubunit DNA replication complex, not being directly essential for DNA synthesis but having an important structural role in the complex, with alterations in the protein leading to instability of the complex and the observed defect in DNA replication. When antibodies to the uracil DNA glycosylase become available, it should be possible to perform immunoprecipitations of the uracil DNA glycosylase to screen for associated proteins. Similarly, the identification of extragenic suppressors of VV ts4149 within other viral genes would provide strong evidence for the complexing of the VUG and SUG gene products with other viral polypeptides.

Now that we have identified a poxvirus uracil DNA glycosylase, an important question to be resolved concerns how the complete excision repair pathway proceeds within the infected cell cytoplasms. Poxviruses induce the synthesis of DNase activities (28, 45) which could function as AP endonucleases, or alternatively, a host cell endonuclease might be recruited to create the nick required to initiate repair. Presumably, the viral DNA polymerase is then involved in the repair synthesis, which could be completed by the virus-encoded DNA ligase. A reconstitution of this repair process should be possible with the expression of poxvirus DNA polymerase and the identification of the genes encoding the viral nucleases. Of particular interest will be the elucidation of the apparent linkage between this repair enzyme and the components of the DNA replication apparatus.

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