
Identification, physical map location and sequence of the *denV* gene from bacteriophage T4

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ABSTRACT

The *denV* gene from bacteriophage T4, which codes for endonuclease V, a small DNA repair enzyme, has been cloned and identified by an approach combining DNA sequencing and genetics, independent of the phenotypic effect of the cloned gene. Appropriate DenV⁺ and DenV⁻ deletion mutants were mapped physically to define precisely a region encompassing the *denV* gene. This region was sequenced in order to identify a protein-coding sequence of the correct size for the *denV* gene (400-500 bp). Finally, identification was confirmed by sequencing the corresponding fragments cloned from four genetically and phenotypically well-characterized *denV* mutants. The *denV* gene is located at 64 kb on the T4 genome, adjacent to the *ipII* gene, and codes for a basic protein of 138 amino acids with a deduced molecular weight of 16,078.

INTRODUCTION

The *denV* gene of bacteriophage T4 confers enhanced resistance to ultraviolet light (UV) and is largely responsible for the difference in UV resistance between T4 and the other T-even phages (1). The gene codes for endonuclease V, a 16,000 dalton excision repair enzyme (2) with pyrimidine dimer-DNA glycosylase and apyrimidinic-site endonuclease activities (2,3) which act sequentially to introduce a nick into the damaged strand at the site of the lesion, leaving the pyrimidine dimer attached to the DNA by a single glycosidic bond on the 3'-side of the nick (3). Although endonuclease V has no catalytic activity against unirradiated DNA (4), its processive mode of action suggests that it is able to bind to undamaged DNA (5). The enzyme has also been reported to be involved in heteroduplex recombination (6) and packaging of phage DNA (7) in T4-infected *Escherichia coli*.

Experiments with permeabilized *E. coli* cells have shown that endonuclease V is able to complement defects in the endogenous *uvrA,B,C* excision repair system (8). More remarkably, endonuclease V has been reported to restore excision repair in permeabilized (9,10) or disrupted (11) human fibroblasts from most of the complementation groups associated with the hereditary UV-repair deficiency, xeroderma pigmentosum. The *denV* gene is thus a good

candidate for restoration of excision repair of UV damage in both prokaryotic and eukaryotic cells.

Lloyd and Hanawalt (12) reported conversion of *Uvr*⁻ *E. coli* cells to wild-type levels of UV resistance by transformation with plasmids containing *Sal*I or *Xho*I restriction fragments from the *denV* region of the T4 genome followed by selection of the transformants with UV irradiation. The plasmids isolated by this procedure were not extensively characterized and were not shown to transfer UV resistance except with concomitant reselection with UV. After repeated unsuccessful attempts to clone the gene from purified T4 DNA fragments by UV selection, we adopted an approach independent of the phenotypic effect of the cloned gene, relying instead on a combination of DNA sequencing and classical genetics. Here we describe the successful identification of the *denV* gene by this approach.

MATERIALS AND METHODS

Strains of T4 and *E. coli*

T4 phage DNA containing cytosine (dC-T4) instead of glucosylated hydroxymethyl cytosine (GHMC) was obtained from a *56 denA denB alc8* T4 strain grown in *E. coli* strains K803 and B834, all gifts of R. Marsh (13). T4 strains *farP12*, *farP13*, and *farP23* were kindly provided by D. Hall (14) and were grown in *E. coli* CR63. All other T4 strains were grown in *E. coli* CR63 or *E. coli* B, both provided by D. Hall. Wild-type T4D, *uvs5(ΔdenV)*, *uvs13(ΔdenV)* (15,16), and *F431(tsdenV)* (17) were gifts of M. Sekiguchi. *T4v1* was obtained from W. Harm (1). Other *E. coli* strains were supplied by B. Bachmann from the *E. coli* Genetic Stock Center at Yale University.

Restriction and Cloning of T4 DNA

dC-T4 DNA for cloning or for Southern blotting was digested with one or more restriction enzymes (New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratory) according to the manufacturer's recommendations. Because the mutations responsible for incorporation of C in place of GHMC in this DNA were leaky in our hands, digests of dC-T4 DNA were seldom complete, although in most cases complete digestion products predominated. GHMC-T4 DNA was digested either partially or to completion with *Taq*I, *Eco*RV, or *Aha*III. Fragments from complete digests to be used as hybridization probes, or to be cloned in pBR322 (18), were recovered from agarose or acrylamide gels by electro-elution into dialysis bags, and when necessary, purified free of soluble gel material by DE52 chromatography (19). Partial digests for cloning were size-selected on sucrose gradients (19) and the fraction corresponding to

4-6 kb was retained for "shotgun" cloning in pBR322. Fragments with blunt or cohesive ends were ligated into linearized pBR322 vectors as described by Maniatis *et al.* (19), and the ligation mixture was used to transform (20) HB101 or AB2480 (UvrA⁻, RecA⁻) *E. coli* cells. Antibiotic-resistant colonies were screened by filter hybridization with the gel-purified 15 kb (58-73 kb on the T4 map) SalI T4 fragment or with a 6 kb (58-64 kb) SalI - XhoI fragment derived from it (25). Plasmids extracted from positive colonies were mapped on the T4 genome by Southern blotting (21) experiments, in which T4 DNA was digested with SalI, XhoI, XbaI, PstI, SalI + XhoI, EcoRI, HindIII, and TaqI, and hybridized to ³²P-labeled plasmid DNA; alternatively, plasmid DNA was digested with various restriction enzymes and hybridized to gel-purified T4 DNA fragments.

UV-Survival Curves

UV resistance associated with wild-type, v1, and farP strains of T4 bacteriophage was determined by plotting survival curves of irradiated virus. Dilutions of T4 stocks in phosphate-buffered saline (PBS) were irradiated at 254 nm as 100 μ l droplets in a culture dish, using a fixed Mineralight UV lamp calibrated with a Black-Ray photometer (both from Ultraviolet Products, Inc.). Aliquots were removed after each irradiation and plated with *E. coli* CR63, and plaques were counted the next day.

Physical Mapping of farP Deletion Mutants

T4 phage stocks from the indicated strains were dotted onto a lawn of *E. coli* CR63 and incubated overnight. Phage from the resulting cleared spots were transferred to nitrocellulose filters by blotting and hybridized (22) to various ³²P-labeled probes obtained by nick-translation of DNA fragments eluted from polyacrylamide gels. The filters were then washed repeatedly with hybridization buffer, dried, and exposed to x-ray film (Kodak XAR-5).

DNA Sequencing

DNA sequences were determined by Maxam-Gilbert cleavage (23) of end-labeled fragments derived from cloned plasmids containing T4 DNA. Fragments with 3'-recessed ends were labeled by extension with the Klenow fragment of DNA polymerase I, using α ³²PdATP or α ³²PdCTP and appropriate unlabeled deoxy-nucleotide triphosphates.

RESULTS

Physical Mapping of the denV Gene with Deletion Mutants

On the basis of genetic crosses, the denV gene had been placed between the regB gene and the ipII gene, at approximately 62 kb on the physical map

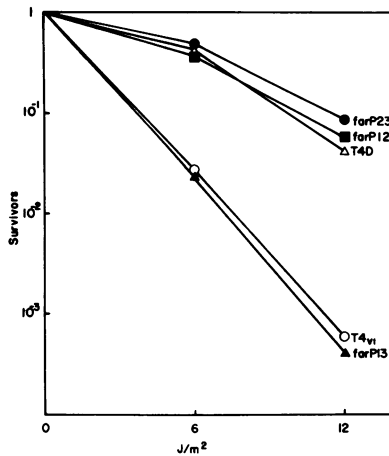


Figure 1. UV-survival curves of $DenV^+$ and $DenV^-$ deletion mutants. Dilute stocks of wild-type, T4v1, and farP strains were irradiated and plated for counting as described in Materials and Methods.

(24). However, neither the genetic mapping data nor the assigned location of the reference genes on the physical map seemed reliable enough to form the basis for a major sequencing effort. Accordingly, we first undertook physical mapping of the *denV* gene, using the deletion mutants *farP12*, *farP13*, and *farP23*, which are missing both the *tk* gene and the *regB* gene (14), located approximately at 60 kb and 61 kb, respectively, on the physical map (24,25). UV-survival curves for these three strains (Figure 1) show that *farP12* and *farP23* resemble the wild-type T4D strain, while *farP13* resembles the well-characterized $DenV^-$ strain T4v1 (1), confirming the observation of D. Hall (personal communication). The end-points of these deletions were mapped by dot-blot hybridization with a variety of restriction fragments from the 60-65 kb region on the T4 genome (Figure 2B). Autoradiograms of these dot blots and the resulting deletion maps are shown in Figures 2A and 2B, respectively. In all three strains, sequences to the left of 61.7 kb on the restriction map (Figure 2B) are missing, as expected from the established $RegB^-$ phenotype. The right-hand end-points of the deletions in the $DenV^+$ strains *farP12* and *farP23* are both between 61.7 kb and 62.1 kb on the restriction map. The deletion in the $DenV^-$ strain *farP13* extends 2-3 kb further, ending between 64.0 kb and 64.8 kb. These results indicate that the *denV* gene cannot extend to the left of the *AhaIII* restriction site at 61.7 kb, and that either the gene or an essential control element must fall at least partly to

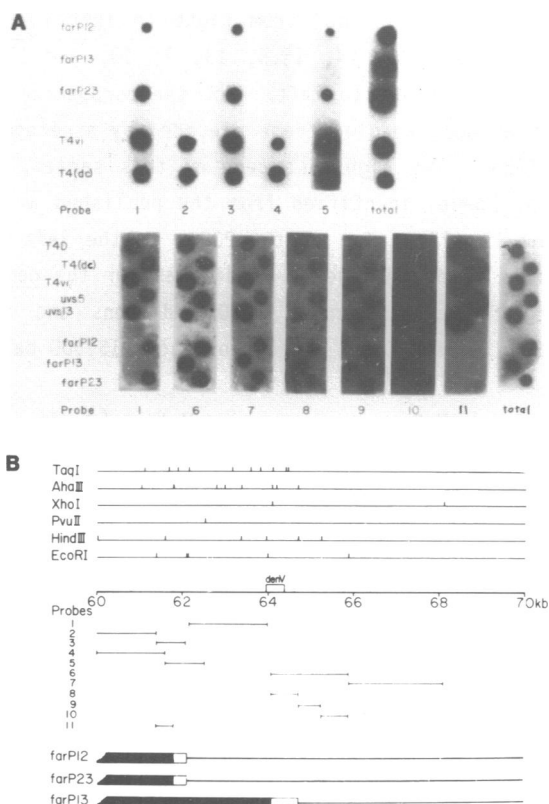


Figure 2. Mapping of DenV⁺ and DenV⁻ deletions. Phage stocks were spot blotted and hybridized to T4 probes as described in Materials and Methods. A) Autoradiogram of spot blots. The numbers refer to probe fragments shown on the accompanying map. B) Restriction map of the 60-65 kb region of the T4 genome, showing the location of the fragments used as hybridization probes, the extent of the *farP* deletions, and the location of the *denV* gene. Probes 1-5 and 11 were derived from cloned fragments resulting from partial *Hind*III or *Eco*RI digests of T4 DNA; probes 6-10 were derived from a gel-purified 4 kb *Xho*I fragment. The probes were: 1, 1.8 kb/*Eco*RI; 2, 1.9 kb/*Eco*RI; 3, 0.7 kb/*Eco*RI; 4, 1.5 kb/*Hind*III; 5, 0.95 kb/*Pvu*II-*Hind*III; 6, 1.8 kb/*Xho*I-*Eco*RI; 7, 2.2 kb/*Eco*RI-*Xho*I; 8, 0.7 kb/*Xho*I-*Hind*III; 9, 0.6 kb/*Hind*III; 10, 0.6 kb/*Hind*III-*Eco*RI; 11, 0.4 kb/*Eco*RI-*Aha*III; total dC-T4 DNA.

the left of the *Hind*III restriction site at 64.8 kb.

Identification of *denV* Gene Candidates by Size

We have determined the sequence of a continuous stretch of nearly 6000 bp (59-65 kb) of the T4 genome (manuscript in preparation), encompassing the region defined by deletion mapping of the *denV* gene (above). We identified eight, closely spaced, protein-coding sequences (PCS's) in the region from

61.7 kb to 64.8 kb, all transcribed from right to left in Figure 2B, coding for proteins of about 11, 16, 24, 15.5, 13, 7, 10, and 11 kilodaltons, in order of occurrence from right to left. For the purpose of this analysis, a PCS was defined as an open reading frame preceded by a recognizable ribosome-binding sequence (26). The rightmost gene of this series, located at 64.7-64.4 kb, is the ipII gene, identified from the published amino acid sequence of internal protein II (27). Two of the PCS's to the left of the ipII gene are the correct size to be considered candidates for the denV gene: PCS16.1 (Figure 3), coding for a protein of 16,078 daltons and located at 64.3-63.9 kb; and PCS15.5, coding for a protein of about 15,500 daltons and located at 63.2-62.8 kb.

Identification of the denV Gene by Sequencing Mutant Strains

PCS16.1 and PCS15.5 were both recloned as TaqI fragments from each of three mutant strains carrying well-characterized denV structural gene mutations: uvs5 and uvs13 (15,16), both of which are amber DenV mutants; and F431 (17), a temperature-sensitive DenV mutant which produces a thermosensitive endonuclease V protein. The sequence of PCS15.5 was found to be identical to that of the wild type in uvs5 and F431. In uvs13, it was found to differ from the wild-type sequences only in that it contained a frameshift (A₅ to A₆) which brings a TAA (opal) stop codon into the reading frame (data not shown). It is not certain whether this change is a cloning artifact or represents an actual difference in this strain, but in any case, it could not give rise to the amber phenotype. On the basis of the sequences from the three mutant strains, we conclude that PCS15.5 can be ruled out as a candidate for the denV gene.

In contrast, PCS16.1 was found to have the expected base changes in all mutant strains examined. The sequence ladders are shown in Figure 4 and the base changes are noted on the sequence in Figure 3. The mutations in uvs5, uvs13 and F431 are G/C to A/T transitions, as expected for mutations induced by hydroxylamine, which reacts specifically with C to cause transitions (28). In uvs5, a C in codon 91 of the wild type has been replaced by a T, converting the a CAG codon (glutamine) to the amber stop codon TAG. Interestingly, the base which has been mutated in this strain is the interior C of an EcoRII restriction site (CCAGG), which is methylated in Dcm⁺ strains of E. coli (29) (Figure 4a) and is thus thought to be a hot spot for C to T transitions in such strains (30). In uvs13, a G in codon 128 has been replaced by an A, converting the TGG codon (tryptophan) to a TAG. The finding of these exact codon changes in two independent amber DenV mutants confirms that the DenV⁻ pheno-

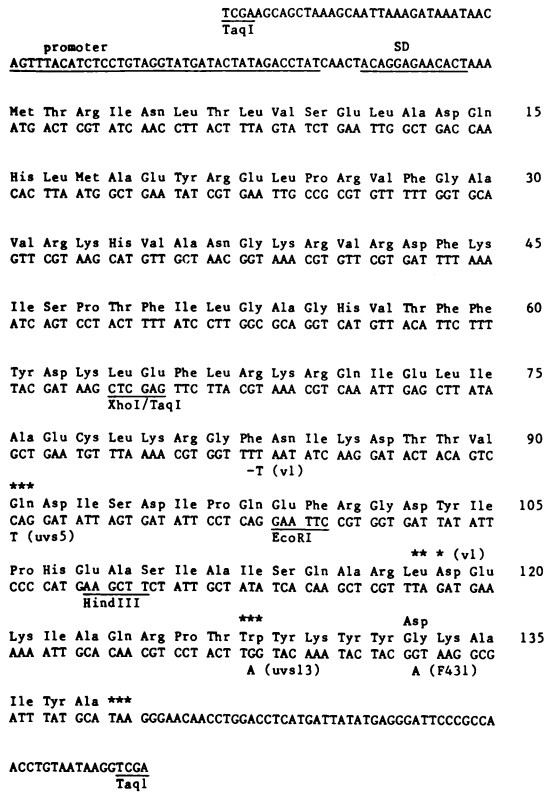


Figure 3. Sequence of two adjacent TaqI restriction fragments containing the denV gene (PCS16.1). The sequence was determined from fragments labeled at the EcoRI, XhoI, and TaqI sites indicated in the figure. All map coordinates have been standardized to the XhoI site, which we have placed at 64.1 kb on the T4 physical map. The internal HindIII site is also indicated. The wild-type sequence is shown, with the base replacements encountered in the mutant strains written underneath. The predicted amino acid sequence is written above the DNA sequence. The underlined 5'-flanking sequences are the proposed promoter sequence (39) and the Shine-Dalgarno sequence (SD) involved in ribosome binding of the mRNA (26).

type results from truncation of the protein product of PCS16.1, although this evidence cannot rule out the possibility that PCS16.1 codes for a positive control element rather than for endonuclease V itself. However, this possibility is rendered exceedingly remote by the base change found in the temperature-sensitive strain F431, where a G in codon 133 has been replaced by an A, converting the GGT codon (glycine) to a GAT codon (aspartate). Since this strain is known to produce a normal amount of protein with altered struc-

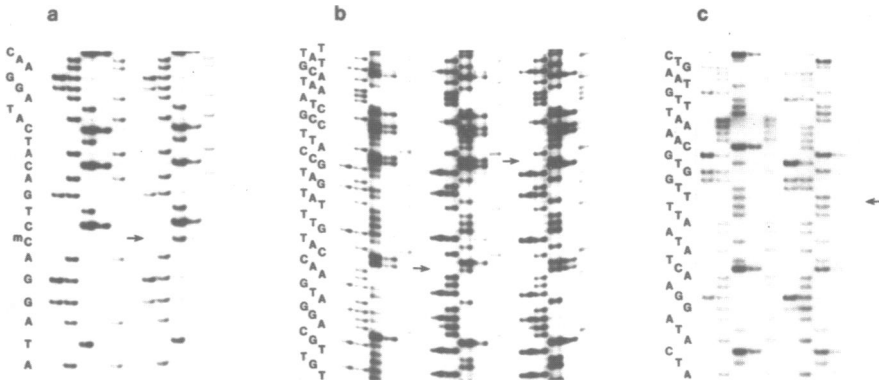


Figure 4. Autoradiograms of sequences of portions of the *denV* gene from wild-type and DenV mutant strains. Derivatives of pBR322 containing the 283 bp *TaqI* fragment encoding the carboxyl-terminal half of the gene (Figure 3) were cut with *EcoRI* and labeled by 3'-end extension, and the 104 bp or 179 bp *TaqI* subfragments were sequenced by Maxam-Gilbert cleavage. The sequencing channels are G, G+A, C+T, C, and A>C, from left to right.

a. Autoradiogram of a 20% polyacrylamide sequencing gel of the 104 bp *EcoRI-TaqI* fragment from wild-type (left) and *uvs5* DNA. The sequence is of the coding (mRNA) strand with the 3'-end at the bottom of the gel. The mutation in *uvs5* is indicated with an arrow. The wild-type sequence has a blank at the position of the base change, because 5-methylcytosine does not react in the Maxam-Gilbert procedure (43) (see text).

b. Autoradiogram of an 8% polyacrylamide sequencing gel of the 179 bp *EcoRI-TaqI* fragment from wild-type (left), *uvs13* (center), and F431 DNA. The sequence is of the non-coding (template) strand, with the 3'-end at the bottom of the gel. The mutations are shown by arrows.

c. Autoradiograms of an 8% polyacrylamide sequencing gel of the 104 bp *EcoRI-TaqI* fragment from wild-type (left) and T4v1 DNA. The position of the deletion in T4v1 is indicated with an arrow.

tural stability (17), the mutation is expected to be in the structural gene itself. The replacement of glycine with aspartate might be expected to alter the charge properties and hence the stability of the protein.

Using the same approach, we have also identified the mutation in the T4v1 strain (1), a deletion of a single T in codon 83, resulting in a frameshift mutation (Figures 3 and 4c). The new reading frame has an amber (TAG) stop codon 100 bp downstream from the site of the mutation. The resulting protein of about 13,500 daltons would be expected to have a normal amino-terminus through amino acid 82, attached to a hydrophobic tail of 33 amino acids.

DISCUSSION

Based on all the lines of evidence here - the location of the PCS16.1 gene within a defined span, the size of the gene, and the verification of the predicted base changes in four strains with structural gene mutations - we conclude that PCS16.1 is the denV gene. Our sequencing evidence alone does not constitute formal proof, since it is conceivable that the base changes detected in PCS16.1 are coincidental and unrelated to the DenV mutant phenotypes of the strains in which they occur. However, we consider this possibility to be extremely remote, particularly in light of the corroborating evidence of size and location. The strategy employed here to identify the cloned denV gene may be of general applicability for cloning genes from genetically well-characterized organisms, particularly in cases where uncertainty as to the phenotypic effect of the cloned gene rules out other selection or screening procedures.

The location of PCS16.1 is over 2 kb away from the previously published map location of the denV gene (24). Our assignment is consistent with observations of differences in the sequence organization of T4 and T2 DNA. Heteroduplex mapping studies indicate that the two genomes are closely homologous in this region but that T2 lacks 1.25 kbp in the region 64.0-65.25 kb (31). In agreement with this, mapping by Southern blotting (21), using cloned T4 DNA probes, has confirmed that although the T2 restriction pattern is similar to the T4 pattern in this region, T2 lacks a block of about 1.2-1.4 kb, somewhere between 64.0 and 65.5 kb on the T4 genome (32). It is likely that the missing DNA includes all or part of the denV gene, accounting for the absence of this gene in T2 (1). The mutant strain T4v1 shows the same UV sensitivity as T2 (1). However, using the Southern technique, T4v1 does not show any detectable difference when compared to wild-type T4 in this region, supporting our finding by sequencing that T4v1 only has a one-base deletion in the denV gene (32).

The amino acid sequence of endonuclease V can be deduced from the DNA sequence of the denV gene and is included in Figure 3. As expected for a DNA-binding protein, endonuclease V is moderately basic, with a net charge of +5 and an unusual abundance of arginine residues (12 out of 138 residues, compared to 10 lysine residues and 17 acidic residues). Five of the arginine residues are adjacent to lysine residues, forming several concentrations of positive charge in the amino-terminal half of the molecule. Preferential codon usage for arginine (12/12 encoded by CGT) as well as for other amino acids is consistent with the pattern reported for genes expressed at a high

level (33), suggesting that it may be possible to obtain high levels of expression of the cloned gene.

The base changes found in several of the mutant strains, together with their functional properties, afford some insight into the molecular basis of endonuclease V activity. The tryptophan which is affected by the mutation in uvs13 is a part of the unusual sequence trp-tyr-lys-tyr-tyr. Suppression of uvs13 in *E. coli* CR63 (SupD), which inserts serine at the TAG codon (34), results in partial restoration of glycosylase but not endonuclease activity (16), while suppression of uvs5 in this strain restores both activities (16). Trp 128 may thus be part of the endonuclease catalytic site. Apurinic and apyrimidinic-site endonuclease activity has been reported for simple model tripeptides of the structure lys-trp-lys (35-37) and lys-tyr-lys (36). These observations further support the evidence already reported (2,3), that the denV gene codes for a protein that has both an endonuclease as well as a glycosylase activity. The T4v1 protein, which presumably has a normal amino-terminal sequence through amino acid 82, has been reported to interfere competitively with the action of wild-type endonuclease V in mixed infections (38). The simplest explanation for this observation is that the T4v1 protein binds to and blocks substrate sites, suggesting that the amino-terminal portion of the molecule may be sufficient for DNA binding.

Our findings suggest a number of possible explanations for our difficulty in cloning the denV gene by conventional approaches. The region around 61 kb probably contains one or more deleterious genes, since we have been unable to clone the 15 kb SalI or XhoI fragments spanning this region (25). Moreover, in repeated experiments no clones spanning this region have been isolated from T4 libraries generated by partial digestion with EcoRI, HindIII, or TaqI; and cloned small fragments such as the 0.7 kb EcoRI fragment at 61.4-62.1 kb (Figure 2B) seem to be susceptible to rearrangement *in vivo* (32). The denV gene itself contains restriction sites for EcoRI, HindIII, and XhoI/TaqI; if these are preferential sites, even partial digests with these enzymes might not contain many copies of the intact gene. The sequencing data reported here were obtained from cloned EcoRI, HindIII, and TaqI fragments containing incomplete copies of the gene.

On the other hand, the structure of the gene itself does not suggest anything exceptional about its expression. The 5'-flanking sequence has an adequate ribosome-binding sequence (6 bases complementary to the 3'-terminal 16S rRNA-binding sequence) (26), and a promoter region with good homology to the -35 *E. coli* consensus sequence (TGTTGACA) (39), but with a slightly

unusual sequence at the presumed -10 position (ATACTAT), which is similar to a T4 late promoter consensus sequence (40). However, the denV gene is reported to be expressed early in the T4 lytic cycle (41), and our results show that it is transcribed from the early (L) strand (42), indicating that the denV gene falls under the early gene category of T4 genes, but might have some promoter features allowing expression later during infection.

Recent results from our laboratory with intact copies of the denV gene cloned in a variety of vectors confirm that it is capable of conferring UV resistance on UV-sensitive E. coli host strains (manuscript submitted). Further characterization of these UV-resistant clones is underway to determine the effect of the denV gene on E. coli DNA repair and DNA metabolism.

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