Facilitated diffusion along nontarget DNA is employed by numerous DNA-interactive proteins to locate specific targets. Until now, the biological significance of DNA scanning has remained elusive. T4 endonuclease V is a DNA repair enzyme which scans nontarget DNA and processively incises DNA at the site of pyrimidine dimers which are produced by exposure to ultraviolet (UV) light. In this study we tested the hypothesis that there exists a direct correlation between the degree of processivity of wild type and mutant endonuclease V molecules and the degree of enhanced UV resistance which is conferred to repair-deficient *E. coli*. This was accomplished by first creating a series of endonuclease V mutants whose *in vitro* catalytic activities were shown to be very similar to that of the wild type enzyme. However, when the mechanisms by which these enzymes search nontarget DNA for its substrate were analyzed *in vitro* and *in vivo*, the mutants displayed varying degrees of nontarget DNA scanning ranging from being nearly as processive as wild type to randomly incising dimers within the DNA population. The ability of these altered endonuclease V molecules to enhance UV survival in DNA repair-deficient *E. coli* then was assessed. The degree of enhanced UV survival was directly correlated with the level of facilitated diffusion. This is the first conclusive evidence directly relating a reduction of *in vivo* facilitated diffusion with a change in an observed phenotype. These results support the assertion that the mechanisms which DNA-interactive proteins employ in locating their target sites are of biological significance.

The problem of how DNA-interactive proteins locate their target sites has been the subject of great interest. The ability of proteins to locate a specific sequence or structural aberration within a large excess of nontarget DNA is crucial in regulating such cellular functions as gene expression, initiation of DNA synthesis, genetic recombination, restriction/modification, and DNA repair. It has been demonstrated that the efficiency of the target search can be increased by reducing its dimensionality (Adam and Delbrück, 1968). A number of DNA-interactive proteins employ a one-dimensional facilitated diffusion process, termed sliding or scanning, to facilitate in locating their targets (reviewed by Lohman, 1986; Ptashne, 1986; von Hippel and Berg, 1989; Mazur and Record, 1989). Scanning or sliding is electrostatic in nature, involving interactions between the acidic phosphodiester backbone of DNA and basic amino acids or regions of the protein. Because the interaction is electrostatic, it is sensitive to the salt concentration of the reaction such that at high concentrations of monovalent salt, the mechanism by which proteins locate specific DNA sequences becomes a three-dimensional search (Leirmo et al., 1987). Sliding has been proposed for the Escherichia coli lac repressor (Riggs et al., 1970; Berg et al., 1988; Winter and von Hippel, 1981; Winter et al., 1981; Barkley, 1981), the restriction enzyme EcoRI (Jack et al., 1982; Langowski et al., 1983; Ehbrecht et al., 1985; Terry et al., 1985), RNA polymerase (Belinstev et al., 1980; Hannon et al., 1980; Park et al., 1982; Roe and Record, 1985; Wheeler et al., 1987; Singer and Wu, 1987, 1988), the bacteriophage λ cro protein (Kim et al., 1987), the Micrococcus luteus UV endonuclease (Hamilton and Lloyd, 1989), the BamHI endonuclease, and the BamHI methylase (Nardone et al., 1986).

T4 endonuclease V is yet another DNA-interactive protein which utilizes a one-dimensional diffusion mechanism to locate its target. Endonuclease V is an enzyme specific for initiating repair at cyclobutane pyrimidine dimers in UV-irradiated DNA. The repair mechanism is comprised of two incision activities: a DNA glycosylase that cleaves the 5' glycosylcylic bond of a pyrimidine dimer, and an apyrimidinic endonuclease that cleaves the 3' phosphodiester bond at the newly formed apyrimidinic site (Haseltine et al., 1980; Gordon and Haseltine, 1980; Radany and Friedberg, 1980; Seawell et al., 1980; McMillan et al., 1981; Nakabeppu and Sekiguchi, 1981; Warner et al., 1981; Nakabeppu et al., 1982). The two activities are separable, and at such, both pyrimidine dimers and apurinic/apyrimidinic (AP) sites in DNA can serve as substrates for the enzyme. At monovalent salt concentrations below 40 mM, T4 endonuclease V locates pyrimidine dimers in DNA by a facilitated diffusion mechanism (Lloyd et al., 1980; Ganesan et al., 1986; Gruskin and Lloyd, 1986). Thus, the enzyme scans nontarget sequences and processively incises at all dimers within the same plasmid duplex DNA prior to dissociating from that DNA molecule. This will be referred to as a processive nicking mechanism. Above 40 mM monovalent salt, the search is expanded to three dimensions due to a severe diminution of protein-nontarget DNA interactions. This will be referred to as a distributive nicking mechanism. Although the physiological salt concentration in *E. coli* is significantly above 40 mM, Gruskin and Lloyd (1988a) have demonstrated that endonuclease V functions processively on UV-irradiated plasmid DNA within *E. coli*. 
Previous studies in this laboratory demonstrated that arginine 3 of endonuclease V was in part involved in the electrostatic interactions necessary for the enzyme to scan DNA (Dowd and Lloyd, 1989a, 1989b). When this amino acid was changed to a glutamine, the mutated enzyme retained catalytic activity, but was no longer capable of scanning DNA in vivo. This loss of (or reduction in) processivity was correlated to the inability of repair-deficient cells to survive UV-irradiation.

This study focuses in more detail on mutations in another region of the enzyme. Secondary structural predictions using the algorithms developed by Cohen et al. (1983, 1986) and Richardson and Richardson (1988) reveal a putative α-helix from amino acids 23 to 39, which contains 4 basic residues on one face of the helix.1 We chose to change two of these amino acids, Arg-26 and Lys-33, to glutamines. These changes were made independently and simultaneously. The mutant enzymes were analyzed for enzymatic activity and for their capacity to scan DNA in vitro and in vivo. These results were then directly related to their ability to confer enhanced UV-resistance to repair-deficient E. coli.

MATERIALS AND METHODS

Oligonucleotide Site-directed Mutagenesis of den V—Mutagenesis of the denV gene, encoding endonuclease V was performed in the baculovirus M13 as described by Dowd and Lloyd (1989a). The sequences of the mutagenic oligonucleotides were designed from the published denV sequence (Radany et al., 1984; Valerie et al., 1984), and encoded the change from Arg-26 (CGT) to Gin-26 (CAA), Lys-33 (AAG) to Gin-33 (CAA), or both mutations simultaneously. The mutant gene was subcloned into the E. coli vector pGX2608 (Recinos and Lloyd, 1986) behind the hybrid λ OPl promoter.

Determination of Intracellular Accumulation of the Mutant Endonuclease V Proteins—Quantitation of endonuclease V protein in both total and soluble protein was as described by Dowd and Lloyd (1989b). Surval Following Ultraviolet Irradiation—AB2480 E. coli (recA::uraA) harboring pGX2608-denV–, pGX2608-denV+, or mutant constructs were grown at 30°C to confluence in LB medium containing 100 µg/ml ampicillin, conditions previously shown to yield maximum accumulation of the enzyme (Recinos et al., 1986). The cells were then diluted in growth medium, spread onto plates containing LB agar supplemented with 100 µg/ml, irradiated at 2.5 mW/cm² for increasing times, and incubated for 36 h at 30°C in the dark. Survival was then measured as colony-forming ability. Three measurements were taken at each point and averaged.

Dimer-specific Nicking Activity—[H]Br322 was irradiated by 254-nm UV light for a total dose of 348 J/m² in order to generate 20–25-thymidylate dimers. The DNA was diluted to 0.05 mg/ml in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 10 mM KCl. Soluble cellular extract containing endonuclease V was added to 1.0 µg of [H]Br322 and incubated at 37°C. The reaction was stopped by addition of electrophoresis loading buffer (50% sucrose, 2% SDS, 50 mM Tris-Cl (pH 8.0), 20 mM EDTA, 0.01% bromphenol blue). The reaction products were resolved by electrophoresis through a 1% agarose gel, and the topological forms of DNA were visualized with ethidium bromide. DNA forms I, II, and III were excised and placed in scintillation vials with 100 µl 1 N HCl. The agarose was melted, 10 ml of aqueous scintillation fluid was added to each vial, and radioactivity was determined by scintillation counting.

AP-Endonucleolytic Activity—Acid depurinated [H]Br322 DNA was prepared as a substrate for the enzymatic activity of endonuclease V as described (Dowd and Lloyd, 1989a, 1989b). The depurinated DNA was solubilized in 10 mM Tris-Cl (pH 8.0), 1 mM EDTA, and 10 mM KCl (0.05 mg DNA/ml solution). The enzymatic reactions proceeded as described for pyrimidine dimer-specific nicking.

Distribution of Incisions in Plasmid DNA During Endonuclease V-initiated Repair—A dimer-specific nicking reaction was performed as described above. Ten nanograms of the wild type or mutant enzymes were used for each measurement. Duplicate samples were taken at each point to be subjected to electrophoresis through a native and a denaturing gel. The reaction was stopped by adding SDS to a final concentration of 1%. Electrophoresis loading buffer was added to the samples to be processed by a native gel. The three forms of the plasmid were separated and analyzed as described above. The samples to be analyzed by denaturing agarose gel electrophoresis were combined with denaturing electrophoresis loading buffer (60% sucrose, 40% glycerol, 60 µg/ml bromphenol blue, 0.05 mg/ml M20) immediately prior to loading the gel in order to minimize nonspecific nicking by the NaOH. Agarose gels (1%) were cast and soaked for 16 h in 30 mM NaOH, 1 mM EDTA (denaturing agarose gel electrophoresis buffer). Samples were subjected to electrophoresis at 40 V for 22 h. The gels were neutralized, and the resulting length distribution of single-stranded DNA was visualized by Southern blot analysis using a nick-translated pBR322 as the probe.

In Vivo Plasmid Repair Kinetic Analysis—The in vivo plasmid repair analysis was performed as described by Gruskin and Lloyd (1988a) with minor alterations. Growth conditions of 40°C in LB medium containing 0.8% glucose were shown previously to result in limiting expression (~300 molecules/cell) of endonuclease V in recA-uraA-denV+ (Gruskin and Lloyd, 1988a). These growth conditions were utilized for the following E. coli strains: AB2480 pGX2608-denV– (wild type or Gin-33). AB2480 harboring pGX2608-denV+ (Gin-26 or Gin-26,33) were grown at 30°C with no additional nutrients. Cells were harvested by centrifugation, and resuspended in one-seventh the original volume of 45 mM NaHPO₄, 22 mM KH₂PO₄, 9 mM NaCl, and 19 mM NH₄Cl. The remaining manipulations were performed in the dark to prevent photoreactivation. The suspension of cells was UV-irradiated at 254 nm at 0°C with rapid stirring for a total dose of 900 J/m². Time course reactions were commenced by the addition of 5 ml of LB at 75°C to 5 ml of the UV-irradiated E. coli suspension at 0°C. The solution was rapidly mixed and then incubated at 37°C. At the specified time points, the 10 ml samples were rapidly frozen in a dry ice-ethanol bath in order to terminate repair activity. The frozen E. coli samples were harvested by a 2-h centrifugation (10,000 × g) at 4°C. The cell pellets were re suspended in 1 ml 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 8% (w/v) sucrose, and 0.5% (v/v) Triton X-100 and incubated 10 min on ice. Lysozyme at 10 mg/ml was added to a final concentration of 1 mg/ml and the suspension was incubated for 10 min on ice. The samples were placed in a boiling water bath for 1.5 min and cell debris and chromosomal DNA was removed by centrifugation at 4°C. The plasmid DNA in the supernatant fraction was precipitated by centrifugation at 4°C. 33P-labeled DNA was resuspended in 0.3 ml of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl.

The plasmid DNA was analyzed by treatment of 40-µl aliquots with a 5-µl aliquot of purified endonuclease V and incubation for 1 h at 37°C. The treatments were terminated by the addition of 25 µl of electrophoresis loading buffer and the samples were analyzed by electrophoresis through a 1% agarose gel (100 V for 5.5 h). The quantitation of [33P]thymidine incorporated in the topological forms of DNA was as described above. This analysis measured the accumulation of endonuclease V-resistant form I DNA.

RESULTS

Accumulation of Mutant Endonuclease V Protein in Cells—The steady-state intracellular levels of wild type and each of the mutants were compared after SDS-polyacrylamide gel electrophoresis and Western blot analysis of total protein in crude cell lysates. Quantitation of endonuclease V protein was also performed on the soluble proteins in cellular sonicates. In both the total and soluble protein analyses, the levels of each of the single mutants (endonuclease V Gin-26 and Gin-33) were 80–100% of the wild type enzyme (data not shown). The level of the double mutant (Gin-26,33) was about one-half of the wild type for both the total and soluble protein analyses (data not shown). Increased protein turnover by intracellular proteolysis due to decreased stabilization by the two amino acid changes may contribute to these lower levels of endonuclease V Gin-26,33.

AP-Endonucleolytic Activity—As mentioned previously,
DNA containing AP sites can serve as a substrate for the phosphodiesterase activity of endonuclease V. When the enzyme cuts the phosphodiester backbone at the AP site, supercoiled (form I) DNA is converted to the nicked circular (form II) DNA. The ability of the mutant enzymes to utilize DNA containing AP sites was measured as a function of enzyme concentration (Fig. 1). Each mutant (Gln-26, open squares; Gln-33, closed squares; Gln-26,33, open circles) retained full AP-endonucleolytic activity at levels equal to or greater than the wild type enzyme (closed triangles).

**Endonuclease V Dimer-specific Nicking Activity**—In a dimer-specific nicking reaction, if the plasmid is initially supercoiled, the reaction generates either form II or form III (linear) DNA. The double-strand breaks are formed if two incisions are made in close proximity and on complementary strands. This assay measures only the first incision, whereas subsequent ones are undetected until a form III-producing incision occurs. Dimer-specific nicking was measured under processive conditions (10 mM KCl) as a function of endonuclease V concentration (Fig. 2). As was observed for AP-specific nicking, the rate of form I DNA loss was more rapid for the three mutants relative to the wild type enzyme (A, closed triangles). This could be explained by either an increased specific activity of the enzyme or by a shift to a more three-dimensional target search, a possibility to be discussed in more detail later. Although each mutant exhibited high levels of nicking, only the Gln-33 mutant (closed squares) produced form III DNA in the reactions (B). However, the levels of form III DNA plateau at an amount less than observed with the wild type enzyme. Endonuclease V Gln-26 (open squares) and Gln-26,33 (open circles) both showed only marginal amounts of accumulated form III DNA.

In order to more fully characterize the dimer-specific nicking activity, a kinetic analysis was performed using equivalent concentrations of the enzymes (Fig. 3). Previously it has been demonstrated that at low salt concentrations (below 40 mM) the wild type enzyme scans DNA incising at each dimer in the plasmid, and form III DNA accumulates in a linear fashion over time (Lloyd et al., 1980; Gruskin and Lloyd, 1986). Also, if the reaction is performed in higher salt concentrations, the target search shifts to a distributive, three-dimensional search. Form III DNA accumulation lags for a short time period under these conditions, until enough random breaks are produced, thereby linearizing the molecule (Gruskin and Lloyd, 1986). This assay monitors the kinetic accumulation of form III DNA and thus is a direct measure of the mechanism of the target search. The Gln-26 (open squares) and the Gln-26,33 (open circles) mutants behaved similarly when nicking was analyzed over time (A). Both mutants exhibited high levels of dimer-specific nicking, but reactions with these enzymes did not result in the accumulation of form III DNA (B). These results suggest that the target search has been shifted from scanning DNA toward a more three-dimensional search (Dowd and Lloyd, 1989a, 1989b). Endonuclease V Gln-33 (closed squares) had the highest level of dimer-specific nicking; and like the wild type, form III DNA did accumulate to significant levels (B). However, for the Gln-33 mutant there did appear to be a very short lag in the accumulation of linear DNA, a result which suggests that the target search could be slightly shifted away from being processive.

To augment this possible lag in form III DNA accumulation, the reaction was slowed by performing it with half the amount of endonuclease V Gln-33 and at 22 °C (Fig. 4, closed squares).
Facilitated Diffusion in DNA Repair

Time (min)

3427

FIG. 3. Kinetic analysis of T4 endonuclease V nicking of form I DNA containing dimers. Cellular lysates containing endonuclease V were added to 1.0 μg of UV-irradiated [3H]pBR322 in 20 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM KCl. Solutions were incubated at 37°C and reactions stopped at the indicated time. Ten nanograms of enzyme were used per point. △, endonuclease V (wild type); ○, Gln-26; □, Gln-33; ▨, Gln-26,33.

Under these conditions, the lag was exaggerated (B) supporting the assertion that the search mechanism has become less processive. Under identical conditions, reactions with the wild type enzyme yielded linear kinetics of form III DNA accumulation (closed triangles).

Effect of KCl Concentration on Standard Time Course Reaction Kinetics—As mentioned previously, the mechanism by which the enzyme locates a pyrimidine dimer can be accurately determined by measuring the sensitivity of the reaction to alterations in the salt concentration. The rate at which the first incision in a plasmid DNA is made by the wild type enzyme continuously increases as the salt concentration of the reaction increases from processive conditions (0 mM salt) to distributive conditions (100 mM salt) (Gruskin and Lloyd, 1986). However, as the salt concentration increases to 200 mM, the rate of the initial incision begins to slow, presumably due to the increased ionic environment competing with the enzyme for interaction with the DNA.3 Under identical conditions, reactions with the wild type enzyme yielded linear kinetics of form III DNA accumulation (closed triangles).

Determination of Incision Frequency in Plasmid DNA during Endonuclease V-initiated Excision Repair—An analysis of the distribution of single-strand breaks at pyrimidine dimer sites within a plasmid DNA population as a function of reaction time is diagnostic of either a processive or a distributive repair mechanism (Gruskin and Lloyd, 1988a; Dowd and Lloyd, 1989a). The accumulation of single-strand breaks and the size distribution of the resulting DNA fragments can be analyzed by denaturing agarose gel electrophoresis. It has been shown previously that at any percentage of form I DNA remaining during a kinetic analysis of dimer incision, the distribution of DNA fragments which are produced by a distributive reaction mechanism are of a significantly larger

3 E. A. Gruskin and R. S. Lloyd, unpublished results.
molecular weight than those observed with a processive reaction mechanism (Dowd and Lloyd, 1989a). The fragments produced by reaction with endonuclease V wild type, Gln-26, Gln-33, and Gln-26,33 were analyzed in this manner to more fully evaluate the mechanism of the target search (Fig. 6). Dimer-specific nicking assays were performed and the reactions were terminated at various time points. Duplicate samples were taken, one for analysis through a native gel to quantitate the level of form I DNA remaining. The second sample was treated for analysis through a denaturing gel in order to determine the distribution of incised dimers within the plasmid population. Those samples which produced equivalent remaining quantities of form I DNA were compared for the size distribution of fragments produced. The wild type, Gln-33, and Gln-26,33 enzymes were compared at form I DNA levels between 33.3 and 34.6% (lanes 1-4, respectively). Wild type, Gln-33, Gln-26, and Gln-26,33 were compared at levels of 22.5-31% form I DNA (lanes 5-8, respectively). Levels of 15.5-17.1% form I DNA were used in the comparison of reactions with wild type (lane 9), Gln-33 (lane 10), and Gln-26 (lane 11). In all the samples, the fragments produced by reaction with the wild type enzyme appeared to be of a smaller size than those observed with the mutant enzymes. Although the reactions performed with endonuclease V Gln-33 produced a spectrum of fragments similar in size to the wild type, the average fragment size was slightly larger. The size of the fragments produced with the Gln-26,33 enzyme was greater than or equal to that observed with the Gln-26 enzyme, and both of these were much larger than those observed with the wild type. It should be noted that in all of the wild type and Gln-33 samples, the level of the form II DNA did not increase above that seen in the untreated DNA (lane 1), a result observed for processive nicking reactions (Gruskin and Lloyd, 1988a, Dowd and Lloyd, 1989a). This is contrasted by the samples of Gln-26 and Gln-33 where form I DNA appeared to be converted to form II DNA, a result observed for a distributive nicking reaction mechanism (Dowd and Lloyd, 1989a).

**Fig. 5.** Consequences of altering the salt concentration on the dimer-specific nicking reaction. Cellular lysates containing ten nanograms endonuclease V/timepoint were incubated at 37 °C with 1 μg of [3H]pBR322 containing dimers. Reaction solution was 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and KCl concentrations were as indicated. Shown is a representative graph; measurements were reproducible to within 10%. A, endonuclease V wild type, 10 mM KCl (●); 100 mM KCl (▲). B, endonuclease V Gln-33, 10 mM KCl (■); 100 mM KCl (▲). C, endonuclease V Gln-26, 10 mM KCl (■); 100 mM KCl (▲). D, endonuclease V Gln-26,33, 10 mM KCl (●); 100 mM KCl (▲).

**In Vivo DNA Repair Kinetic Analysis** - Gruskin and Lloyd (1988a, 1988b) demonstrated that processive and distributive plasmid DNA repair mechanisms can be discerned by analysis of the repair kinetics of the intracellular plasmid DNA pool. The assay, which was developed to monitor *in vivo* plasmid repair, consists of first irradiating cells followed by incubating for various times to allow for repair. Plasmid DNA is then isolated from these cells and monitored for endonuclease V-resistant form I (completely repaired) plasmid molecules. A processive repair mechanism, as observed for endonuclease V, was characterized by a linear accumulation of endonuclease V-resistant form I DNA, the rate of which was inversely proportional to the UV dose. In contrast, the distributive mechanism observed for photolyase was characterized by sigmoidal kinetics of accumulation of endonuclease V-resistant form I DNA. In this case, the time lag observed prior to the accumulation of fully repaired form I DNA increased with increasing UV doses. The kinetics of the accumulation of endonuclease V-resistant form I DNA for a limited processive mechanism had features associated with both distributive and processive processes. This limited processivity was observed for repair initiated by UvrABC in which there was a lag in the accumulation of form I DNA followed by linear kinetics which were also dependent upon the UV dose.

To further define the mechanism of the target search for this set of mutants, the *in vivo* plasmid repair assay was performed (Fig. 7). As expected for the processive mechanism of the wild type enzyme, the accumulation of form I DNA was linear (closed triangles). The kinetics observed for the Gln-33 mutant (A, closed squares) were similar to what would be expected for a limited processive mechanism. There was a lag of approximately ten minutes before the repair kinetics became linear, and the slope of this portion of the graph was the same as exhibited by the wild type enzyme. The repair observed for endonuclease V Gln-26 (B, open squares) was slower than for the wild type or Gln-33, and the kinetics were different. The kinetics of repair were sigmoidal, indicative of a distributive mechanism. The repair kinetics for Gln-26,33
Facilitated Diffusion in DNA Repair

Form II DNA
Form I DNA

**FIG. 6.** The effect of the target-search mechanism on incision frequency. Reactions of 10 ng of wild type and mutant endonuclease V with pBR322 containing 20-25 dimers/molecule were carried out under processive reaction conditions (10 mM KCl). Reaction products were resolved by electrophoresis through a 1.2% denaturing agarose gel (30 mM NaOH). The resulting distributions of single-stranded DNA fragments were visualized by Southern blot analysis using a nick-translated pBR322 probe. These results were reproducible for all samples analyzed at varying amounts of form I DNA remaining. Lane 1, unreacted pBR322; lanes 2, 5, and 9, endonuclease V wild type; lanes 3, 6, and 10, Gln-33; lanes 7 and 11, Gln-26; lanes 4 and 8, Gln-26,33. A, approximately 33.9% form I DNA remaining. Lane 2, wild type, 33.2% form I; lane 3, Gln-33, 34.6% form I; lane 4, Gln-26,33, 33.9% form I. B, approximately 26% form I DNA remaining. Lane 5, wild type, 25.6% form I; lane 6, Gln-33, 22.5% form I; lane 7, Gln-26, 24.8% form I; lane 8, Gln-26,33, 31% form I; C, approximately 16.6% form I DNA remaining. Lane 9, wild type, 17.2% form I; lane 10, Gln-33, 15.5% form I; lane 11, Gln-26, 17.1% form I.

(B, open circles) were also sigmoidal, although the population of fully repaired plasmid DNA was significantly less than observed with Gln 26. It should be noted that if this assay is performed in the absence of endonuclease V, no repair is detected for time up to at least 100 min (data not shown).

**UV Survival as Measured by Colony-forming Ability—Repair-deficient E. coli AB2480 (recA- uvrA-) which had been transformed with plasmids containing the mutant denV genes were irradiated with increasing doses of UV light (Fig. 8).** Colony-forming ability of these cells following UV exposure is a measure of complete in vivo activity, since one unrepaired pyrimidine dimer is lethal to the cell. The expression of both wild type endonuclease V (closed triangles) and Gln-33 (closed squares) produced greatly enhanced UV survival over that of the repair-deficient cells (closed circles). Gln-26 (open squares) provided an intermediate measure of UV survival, and Gln-26,33 (open circles) was slightly less than Gln-26, whereas still providing more resistance to UV light than observed with the parent AB2480 cells. The reduced ability of Gln-26,33 to enhance UV resistance was not due to a reduced accumulation of the enzyme. When expression of the Gln-26 enzyme was modulated to levels equivalent to the Gln-26,33 enzyme, the repair capacity of Gln-26 did not decrease (data not shown).
This study was undertaken to investigate which portions of T4 endonuclease V are responsible for the ability of the enzyme to scan nontarget DNA in search of a pyrimidine dimer. Earlier studies in this laboratory focused on arginine 3 in this regard (Dowd and Lloyd, 1989a, 1989b). Arginine 3 was determined to be involved in scanning DNA in vitro. It was proposed that a loss of this nontarget DNA interaction was linked to the cells' inability to survive UV insult. However, low in vivo repair levels made the direct comparison of in vivo scanning ability and survival impossible for these mutants. In contrast, due to higher levels of in vitro repair demonstrated by the mutants in this study, the direct comparison between the mechanism of the target search and in vivo consequences has been made possible.

Mutations in endonuclease V of Arg 26 → Gln 26, Lys-33 → Gln-33, and Arg-26, Lys-33 → Gln-26, Gln-33 produced enzymes with full enzymatic activities (AP- and dimer-specific nicking), but with varying degrees in facilitated diffusion. The target search mechanism was analyzed in vitro as well as in vivo, and thus the ability to scan nontarget DNA could be compared directly to the ability to enhance cellular survival after UV insult. In vitro, the following parameters are all sensitive indicators of the mechanism of the target search: the kinetics of the accumulation of form III DNA in a dimer-specific nicking analysis, the sensitivity of the reaction to the salt concentration, and the accumulation of single-strand breaks during the reaction. The results of these in vitro analyses can be confirmed by plasmid repair analyses in vivo.

The studies presented in this paper demonstrate that endonuclease V Gln-33 has been affected and the least of this group of mutants. It retained complete in vitro enzymatic activity and was capable of effecting cellular survival after UV insult at levels equivalent to those produced by the wild type enzyme. The fact that the rate of the initial incision increased with increasing salt concentration argues in favor of a processive (one-dimensional) target search. However, further evidence suggests that the nature of the target search has been shifted slightly to a limited processive mechanism, where the number of base scanned or the time associated with nontarget DNA has been slightly decreased relative to the wild type enzyme. In vitro evidence for this limited processivity included the small lag in the accumulation of form III DNA over time and a slight shift in the average DNA fragment size produced toward larger fragments as analyzed by denaturing agarose gel electrophoresis. In vivo, this was supported by plasmid repair kinetics which were consistent with a limited processive mechanism in that a short lag was observed in the repair with subsequent linear kinetics. These results indicate that an enzyme with a slight reduction in protein-nontarget DNA interactions is capable of functioning properly in the cell.

The ability of endonuclease V Gln 26 to interact with nontarget DNA appeared to be affected to a much greater extent. In in vitro dimer-specific nicking reactions, there was no accumulation of form III DNA, and the fragments produced were of a larger size than observed with the wild type enzyme. These results were shown previously to be indicative of a shift to a distributive target search. Finally, the capacity of endonuclease V Gln-26,33 to scan DNA appeared to be the most altered. In the dimer-specific nicking analysis, form III DNA did not accumulate, a result which was consistent with that obtained in the analyses of Gln-26. As the salt concentration of the reaction was raised from 10 to 100 mM KCl, the kinetics became slower, a result in direct agreement with this hypothesis. The single-strand breaks produced during the reaction and the resulting larger size fragments also support this conclusion.

In order to confirm the hypothesis that endonuclease V Gln-26 and Gln-26,33 have their target search mechanisms shifted toward a distributive mode, the in vivo repair of plasmids was investigated. Both enzymes exhibited sigmoidal kinetics in the accumulation of endonuclease V-resistant form I DNA, results expected for a distributive, three-dimensional mechanism of search. The total repair observed for Gln-26,33 was at a lower level than for Gln-26. These results, as well as the sensitivity of the in vitro reaction to salt, suggest that the ability of the double mutant to scan had been affected to a greater degree than either of the single mutants. It may be that the double mutant is a product of the additive effects of the nature of the target search of each of the single mutations and as such has an increasingly difficult time in locating a pyrimidine dimer.

Although the shift from a processive to a limited processive (Gln-33) provided UV resistance equal to levels of the wild type, further shifts away from a one-dimensional target search toward a three-dimensional one bad profound effects on the ability of repair-deficient cells to survive UV irradiation. By changing Arg-26 to Gln, the ability of the enzyme to scan nontarget DNA in vitro as well as in vivo was altered, and this alteration diminished the repair capacity of the enzyme in repair-deficient cells. When Lys-33 was changed in conjunction with Arg-26, the search mechanism was shifted further toward a distributive one, as would be expected if important electrostatic interactions were being neutralized. The in vivo significance of this double mutation was that cells expressing this double mutant were even less capable of enhancing UV resistance than either of the single mutants.

This study has focused on mutations of two basic amino acids on one side of a putative α-helix, residues suspected to be involved in protein-nontarget DNA interactions. The mutations have produced enzymes with repair capacities directly related to their ability to scan the DNA in search of their targets. These results imply that the α-helix may align with the DNA in such a way as to allow electrostatic contact of the backbone with Arg-26 and Lys-33. It appears that Arg-26 provides a more important contact with the DNA, a role possibly aided by the natural dipole of the helix. The possibility exists that these amino acids may not be located on the same α-helix or not on an α-helix at all. Only by the determination of a crystal structure will the location of these residues in the secondary and tertiary structures be known, but in the absence of such a structure, the testing of structural predictions will suffice.

Theory of facilitated diffusion predicts that major and characteristic changes in the association and dissociation kinetics of the protein-target complex may be expected as a result of varying the affinity of the protein for nontarget DNA (Berg et al., 1981). Previously, this has been experimentally addressed only by varying the ionic environment of the system. In this paper, we discuss a different approach to addressing this question, an approach which entails the abolition of key nontarget electrostatic interactions at the protein level. By mutating two basic amino acids in endonuclease V we were able to produce enzymes with varying levels of in vivo and in vitro facilitated diffusion, and these levels were directly related to the levels of total cellular repair. One may speculate on the necessity of scanning by other DNA-interactive proteins. The importance can be appreciated in systems where an enzyme is expressed in limiting quantities and must locate its cognate sequence rapidly in order to maintain cellular integrity. Facilitated diffusion leads to an acceleration in the rate of the productive search, and a more efficient search may
provide an advantage to the cell and as such, be a genetically selected advantage.

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