Measurement of Repair Patch Size by Quantitation of Nucleotides Excised During DNA Repair In Vivo

ERIC H. RADANY AND ERROL C. FRIEDBERG*

Laboratory of Experimental Oncology, Department of Pathology, Stanford University, Stanford, California 94305

Received 7 January 1983/Accepted 3 May 1983

Escherichia coli *uvr*⁻ cells, prelabeled in their DNA, were infected with phage T4 *denV*⁺ or T4 *denV*⁻ under conditions that preclude phage-mediated degradation of the bacterial chromosome. Measurement of the distribution of acid-soluble radioactivity between pyrimidine dimers and nondimer nucleotides in cell extracts yielded calculated estimates of the average size of excision repair tracts that are in good agreement with the size of repair patches determined by others using direct measurement of repair synthesis.

Pyrimidine dimer (PD)-DNA glycosylases are enzymes that can initiate the excision repair of PDs from DNA (4). Such enzymes have been purified and extensively characterized from *Micrococcus luteus* (6, 7) and *Escherichia coli* cells infected with T4 phage having an intact *denV* gene (3, 10-12, 14-16, 19). We have previously demonstrated the action of the phage T4 enzyme in vivo by using an experimental system in which UV-irradiated, repair-deficient (*uvr*⁻) *E. coli* cells are infected with T4 *denV*⁺ phage. During these experiments, we have consistently observed that the proportion of total radioactivity present in the acid-soluble fraction is greater in extracts of T4 *denV*⁺-infected cells than in those of T4 *denV*⁻-infected or uninfected *uvr*⁻ cells, and we have suggested that this finding reflects the excision repair of PDs from chromosomal DNA (15). The average number of nucleotides excised from *E. coli* DNA during each PD excision event can be calculated, and hence the average repair synthesis patch size is estimated to be about six nucleotides (15). However, these calculations have been possibly complicated by the fact that wild-type phage T4 encodes a salvage system that induces degradation of host DNA to an acid-soluble form and promotes utilization of the salvaged mononucleotides for phage DNA synthesis (9, 20).

In the present study, we constructed mutant T4 phage defective in their ability to degrade and reutilize *E. coli* DNA. These strains, T4 RP100 (*nd28 ΔdenB* → *rII amN82denV*⁻) or T4 RP200 (isogenic with T4 RP100, except for the *denV*⁻ allele of the *denV* gene), were constructed from crosses of T4 M1 (*nd28 ΔdenB* → *rII*, kindly supplied by E. Kutter, Evergreen State College, Olympia, Wash.) with T4 *amN82* or T4 *denV*⁻ by standard methods (1). T4 *nd28 ΔdenB* → *rII*

amN82 and T4 *nd28 ΔdenB* → *rII denV*⁻ were constructed by first picking progeny that were suppressor sensitive or UV sensitive, as well as restricted on *E. coli* λ lysogens (*ΔrII*) and unable to propagate in the presence of hydroxyurea (*nd28*) (20). Each of these was backcrossed to T4 M1 twice to obtain T4 RP100 or the *denV*⁻ parent of T4 RP200.

In the experiments, repair-deficient (*uvr*⁻) cells of *E. coli*, labeled in their DNA with [³H]thymidine, were UV irradiated and then infected with T4 *denV*⁺ phage, T4 *denV*⁻ phage, or left uninfected (Fig. 1). During the subsequent incubation, labeled thymine-containing PDs were excised from chromosomal DNA in the T4 *denV*⁺-infected cells, together with some radioactivity associated with monomeric thymine, and became acid soluble. Not unexpectedly, thymine-containing PDs were not detected in the acid-soluble fraction of *denV*⁻-infected or uninfected cells. Figure 1A shows that the formation of acid-soluble radioactivity accompanying excision repair could be readily detected and quantitated as an increase over the background level present in T4 *denV*⁻-infected cells. A number of observations indicate that this increase was due solely to the degradation of DNA associated with the excision repair of PDs. (i) The pair of phage strains used are isogenic except for the *denV* genes; thus, the observed increase in acid-soluble radioactivity was almost certainly due to expression of the *denV*⁺ gene product, which is apparently specific for PDs in DNA (4). (ii) The increment in acid-soluble radioactivity was observed with UV-irradiated but not with unirradiated cells (compare Fig. 1A and B). (iii) During the 12-min period of incubation, the levels of acid-soluble radioactivity were identical in the excision-defective T4 *denV*⁻-infected and unin-

FIG. 1. Excision repair of PDs in the chromosomal DNA of UV-irradiated *uvr*⁻ *E. coli* cells after infection by T4 *denV*⁺. (A) *E. coli* K-12 strain SR143 (W3110 *uvrA6* *thyA* *deoC* *sup*⁻, obtained from K. C. Smith, Stanford University) was radiolabeled with [³H]thymidine as previously described (15). At a cell density of $\sim 2 \times 10^8$ CFU/ml, cells were harvested, suspended in medium supplemented only with unlabeled thymidine, and reincubated at 37°C with shaking for 75 min. Cells were chilled to 4°C and divided into two portions; one portion was irradiated at 4°C with an effective UV dose of 50 J/m² at 254 nm. The unirradiated portion of cells was held at 4°C. Infection with phage was at a multiplicity of 10; some cells were left uninfected. After incubation at 37°C with shaking for 3 min to allow phage adsorption, 1/10 volume of 10× supplements (5% glucose, 1% Casamino Acids) was added to each culture, and incubation was continued 12 min longer. Cells were then harvested and processed for the measurement of nucleotide radioactivity in acid-soluble and acid-insoluble fractions, as described previously (15). The radioactivity in thymine-containing PDs in both fractions was also measured (15). For each culture, three samples of cells were analyzed independently; the proportion of total nucleotide radioactivity in the acid-soluble fraction was calculated for each sample. Open bars, mean of the three values for the percentage of total nucleotide in the acid-soluble fraction for each culture; error bars, $\pm$ standard error of

the mean; solid area, fraction of acid-soluble radioactivity in thymine-containing PDs. No radioactivity in PDs was detected in the acid-soluble fractions from the *denV*⁻-infected or uninfected cells. (B) Unirradiated *uvr*⁻ cells were treated in the same way as the irradiated cells, with the exception that the cultures were incubated for 35 min after the addition of the 10× supplements. Bar definitions are as in (A).

fecting *uvr*⁻ cells (Fig. 1A). This was expected, as the phage used were constructed to be defective in endonucleases II and IV. The defect in these enzymes blocks the breakdown of host DNA during phage infection (9, 20). The radioactivity in acid-soluble thymine-containing PDs constituted about one-half of the total increase associated with excision repair of UV-irradiated *E. coli* DNA. Thus, on the average, the thymine-containing PDs and adjacent nucleotides excised in each repair event contained the same amount of thymine. At the UV dose used in these experiments, the PD species T<>T, T<>C, C<>T, and C<>C were formed in approximately equal numbers, as previously reported (5, 7), so the average PD contains about one thymine. Assuming that the composition of adjacent excised nucleotides reflects that of bulk *E. coli* DNA, any four nucleotides would also contain, on the average, one thymine. Thus, the observed distribution of excised radioactivity between PD and nondimer nucleotides in acid-soluble material is consistent with a patch of six nucleotides excised (and presumably resynthesized) for each PD repair event. This value may, in fact, be an overestimate, as it has been shown (5, 7) that PDs tend to be formed preferentially in short pyrimidine-rich DNA sequences. Thus, the nucleotides excised with PDs should be, on the average, thymine-enriched relative to bulk *E. coli* DNA.

In this analysis, we have assumed that both the PD and nondimer nucleotides excised from chromosomal DNA are stable in the acid-soluble fraction during the period of incubation. This has been previously demonstrated to be true for thymine-containing PDs by showing equality between the radioactivity in PDs lost from the acid-precipitable fraction of DNA during incubation and that recovered in the acid-soluble fraction after incubation (15). The excised non-PD radioactivity is probably substantially stable as well, since the reincorporation of excised nucleotides into acid-insoluble phage DNA is precluded by an amber mutation that abolishes T4 DNA polymerase activity in the nonsuppressing host cells used in these experiments (9). Furthermore, semiconservative *E. coli* DNA replication is blocked in cells that have been heavily UV irradiated (8). It is difficult to exclude the possibility of reincorporation of excised nucleotides into the repair synthesis patches themselves.

the mean; solid area, fraction of acid-soluble radioactivity in thymine-containing PDs. No radioactivity in PDs was detected in the acid-soluble fractions from the *denV*⁻-infected or uninfected cells. (B) Unirradiated *uvr*⁻ cells were treated in the same way as the irradiated cells, with the exception that the cultures were incubated for 35 min after the addition of the 10× supplements. Bar definitions are as in (A).

This seems unlikely however, because the total amount of DNA degradation in these experiments is very small compared with the extent of degradation that is required to cause detectable dilution of exogenous labeled precursor during incorporation into repair synthesis patches in uninfected cells (2). Furthermore, cells were incubated after UV irradiation in a high concentration of unlabeled thymidine (10 $\mu\text{g/ml}$). Finally, it should be noted that there is an excellent agreement between our estimate of repair patch size in T4-infected cells obtained by analysis of the excised material (≤ 6 nucleotides) and that obtained by photolysis of repair patches in T4 DNA labeled with BUdR (~ 4 nucleotides) (21). This quantitative correlation also indicates that all PD excision repair events in T4-infected cells give rise to excised material that is acid soluble. If this were not the case, some excision repair events would not be detected, and our calculations would underestimate the size of excision-resynthesis tracts. This conclusion is consistent with the observation that no excised PDs are detected in the acid-insoluble fraction of T4 DNA after infection of *E. coli* cells with UV-irradiated T4 phage (13).

The measurement of repair patch size described here is technically less laborious than procedures that utilize the incorporation of density labels into DNA or the photolysis of DNA substituted with 5-bromouracil (17, 18). Furthermore, it is potentially applicable to any experimental system in which PDs are retained intracellularly, i.e., are not excreted into the medium. Indeed, in preliminary experiments we have demonstrated the utility of this procedure for measuring repair patch sizes in uninfected repair-proficient *E. coli*. This, as well as most other techniques described previously, yields average repair patch sizes and hence does not discriminate between different classes of repair synthesis where they exist. Nonetheless, measurement of the average patch size is a useful parameter for investigating the effects of genetic and physiological perturbations of DNA excision repair in living cells.

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