Endonuclease from *Micrococcus luteus* Which Has Activity Toward Ultraviolet-Irradiated Deoxyribonucleic Acid:
Its Action on Transforming Deoxyribonucleic Acid

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An endonuclease purified from *Micrococcus luteus* makes single-strand breaks in ultraviolet (UV)-irradiated, native deoxyribonucleic acid (DNA). The purified endonuclease is able to reactivate UV-inactivated transforming DNA of *Haemophilus influenzae*, especially when the DNA is assayed on a UV-sensitive mutant of *H. influenzae*. After extensive endonuclease action, there is a loss of transforming DNA when assayed on both UV-sensitive and -resistant cells. The endonuclease does not affect unirradiated DNA. The results indicate that the endonuclease function is involved in the repair of biological damage resulting from UV irradiation and that the UV-sensitive mutant is deficient in this step. We interpret the data as indicating that the various steps in the repair of DNA must be well coordinated if repair is to be effective.

Crude extracts of *Micrococcus luteus* (*Micrococcus lysodeikticus*) are capable of reactivating ultraviolet (UV)-irradiated, biologically active deoxyribonucleic acid (DNA; 5) and similar extracts can excise UV-induced pyrimidine dimers from DNA in vitro (2). Since cyclobutyl pyrimidine dimers are lesions in many biological systems and the dark repair of UV-irradiated DNA in vivo is associated, at least in part, with the excision of dimers in vitro (6, 10, 14, 17), extracts of *M. luteus* are good material for the isolation of enzymes associated with excision reactions. Such extracts preferentially degrade UV-irradiated DNA, (16) and, when pyrimidine dimers are repaired in vivo prior to extraction of the DNA from cells, there is a loss of sensitivity to such extracts (18). The degradative activity of the extract is apparently partially due to the presence of an endonuclease specific for damaged DNA. The purification of such endonucleases has been carried out by several groups of investigators (3, 8, 19). A previous paper (3) describes the purification and properties of an endonuclease from *M. luteus* which acts preferentially on UV-irradiated, native DNA. The nuclease makes single-strand nicks close to the dimers in the DNA. In this paper, we describe experiments with the purified endonuclease which show that it can reactivate UV-inactivated transforming DNA, especially when the DNA is assayed on a UV-sensitive mutant of *Haemophilus influenzae*. Our results indicate that (1) the specific endonuclease is involved in the repair of biological damage resulting from UV irradiation, probably as the first step in the excision of pyrimidine dimers and (2) that the UV-sensitive *H. influenzae* mutant is deficient in this step. We interpret our data further to indicate that the various steps in the repair of DNA must be well coordinated if repair is to be effective.

**MATERIALS AND METHODS**

UV-endonuclease. The enzyme preparation from cells of *M. luteus* is described in the previous paper (3).

**Estimation of single-strand breaks.** Single-strand breaks resulting from the action of endonuclease on UV-irradiated DNA were estimated as follows (see reference 3). After incubation with the enzyme, the UV-irradiated DNA was denatured in alkali, neutralized, and chromatographed on small columns of Sepharose 4B to separate the undegraded DNA (molecular weight greater than 106) from the pieces resulting from endonuclease action (molecular weights approximately 24,000 for the UV dose used). The DNA used for the estimation of single-strand breaks was 3H-thymidine labeled *Escherichia coli* DNA (160,000 counts per min per µg).

Microorganisms. *H. influenzae* strains used were
Rd, wild-type, and able to excise pyrimidine dimers in vivo, and DB112, a UV-sensitive strain unable to do host-cell reactivation and unable to excise pyrimidine dimers in vivo (11, 12). UV-irradiated transforming DNA gives a considerably lower survival when assayed with DB112 than with the wild type.

**Transforming DNA and transformation.** Markers on the transforming DNA were streptomycin and cathomycin, which confer resistance to 250 μg/ml and 2.5 μg/ml of these drugs, respectively. Transformation methods for cells sensitive to these drugs have been described previously (11).

UV-irradiation of DNA. Transforming DNA of *H. influenzae* was irradiated with a germicidal lamp as previously described (11). The usual average incident fluence was 2,500 ergs/mm² for the routine assays described below.

**Treatment of transforming DNA with extracts of M. luteus.** The reaction mixture contained: 1 μg of irradiated (usually 2,500 ergs/mm² at 254 nm) *H. influenzae* DNA per ml, and 25 μg of unirradiated salmon sperm DNA per ml in 0.01 M β-mercaptoethanol, 0.001 M ethylenediaminetetraacetate, 0.05 M potassium phosphate buffer (pH 6.5), and UV-endonuclease (0.1 to 6 μg of protein/ml). The reaction (0.3 to 1.0 ml) was started by the addition of enzyme, and the mixture was incubated for various times at 37°C. The reaction was stopped by rapid cooling and freezing. Portions of the mixture were thawed, diluted 100- to 1,000-fold to reduce the concentration of endonuclease, and assayed for transformants (10). In some experiments, noted below, the UV dose to the DNA or the concentration of phosphate buffer was different.

For an incident fluence of 2,500 ergs/mm² to the transforming DNA, the number of pyrimidine dimers per nucleotide is about 2.5 × 10⁻³ (15), representing an average mass between dimers of approximately 130,000 daltons. The routine endonuclease assays were carried out with an irradiated *E. coli* DNA at 2.5-fold the DNA concentration used with the transforming DNA, and this DNA had an average mass of 24,000 between dimers. Thus, when the enzymatic reaction is limited by the amount of enzyme, the time to make all the single-strand breaks in the transforming DNA will be approximately 10-fold shorter than the time to make all the breaks in the standard endonuclease assay using *E. coli* DNA.

**RESULTS**

Figure 1 shows typical results of treatment of UV-irradiated transforming DNA with a partially purified endonuclease from *M. luteus*. A number of points are worthy of note. (i) The enzyme has no activity toward the transforming activity of unirradiated DNA. (ii) When the cells used to assay transforming ability are sensitive cells (DB112), treatment with the enzyme results in an increase in transforming ability, and the initial rate of increase depends on the protein concentration. The activity increase is observed in the case of both a UV-sensitive and a resistant marker. Moreover, the rate of biological reactivation is comparable to that expected from the production of chain breaks measured by endonuclease assays (3).

(iii) When the irradiated transforming DNA, after treatment with endonuclease, is assayed on wild-type cells, there is only a slight increase in activity for the sensitive marker, streptomycin, and an actual decrease in observed activity for the resistant marker, cathomycin. The decrease is greater at high protein concentrations. We shall give data and arguments that both the increased activity observed on DB112 and the decrease observed on wild-type cells arise from the same endonuclease activity.

The reactivating effect is observed over a range of UV doses to the transforming DNA, and there is a constant dose reduction for both the streptomycin and the cathomycin markers when titered on DB112. The data for the streptomycin marker are shown in Fig. 2 for reaction conditions that give the plateau level reactivation indicated in Fig. 1.

We have not been able to separate the three easily measurable activities of these endonuclease preparations: the endonuclease activity, the reactivating activity as measured on DB112, and the inactivating activity, as measured on wild-type cells. Figure 3 shows typical heat-inactivation data for these three assays. Obviously all three activities are inactivated at approximately the same rate. The data in Fig. 3 on the effects of heating may be used to estimate the relative survival of the various activities in terms of the average slopes of the curves in Fig. 3a or the times to reach a given level of reactivation (Fig. 3b) or inactivation (Fig. 3c). These estimates may have large errors; nevertheless, the inactivations of each of the three different activities are quantitatively similar (Fig. 4).

The purification scheme for endonuclease activity involves elution from a carboxymethyl cellulose column. In one experiment, endonuclease purified through the diethylaminoethyl step was adsorbed to carboxymethyl cellulose in 0.02 M PO₄. The bulk of the protein, but no endonuclease activity against irradiated DNA, was eluted with 0.05 M phosphate, pH 6.5. The molarity was increased to 0.15 M, and the endonuclease activity was observed to elute slightly behind the major portion of the protein. Figure 5 shows part of this elution pattern. The endonuclease and the biological activities observed on the two types of cells elute at the same position, again indicating our inability to separate one from the others, even though they are well separated from the bulk of the protein.

The relative rates of endonuclease activity and reactivating activity against UV-irradiated trans-
forming DNA, assayed on DB112, were measured at a number of different salt concentrations. The results of these measurements (Fig. 6) are similar, within experimental error, and indicate that under these conditions the important variable is the total salt concentration.

The similar dependence of the three assays—strand breaks, reactivating activity, and inactivating activity—to heat inactivation, column elution, salt concentration and pH (data not shown), and the fact that there are no activities toward unirradiated DNA is a good indication that the three activities are properties of the same protein.

The data in Fig. 1 indicate that the amount of endonuclease reactivation observed on the UV-sensitive strain, DB112, gives a survival level comparable to that observed on the wild-type strain without the action of endonuclease. We used a preparation purified approximately 50-fold more than in Fig. 1 to investigate these
Survival levels more carefully. Figure 7 shows the ability of irradiated DNA after treatment with endonuclease to transform UV-sensitive (DB112) and wild-type (Rd) cells to cathomycin and streptomycin resistance. In the endonuclease assays with 0.1 μg of protein per ml, the reaction went to 50% completion in about 30 min. The reaction mixture for transforming DNA contains about one-tenth the number of lesions as used in the endonuclease assay, and Fig. 7a shows that the biological reactivation is a maximum at approximately 10 min, as is expected from the endonuclease assay. It is obvious that the amount of reactivation depends on the marker, as is also indicated in Fig. 1, and that at low endonuclease levels the UV-sensitive marker, streptomycin, assayed on DB112, is reactivated by the endonuclease so that its final survival is about the same as that measured on wild-type cells in the absence of endonuclease. Endonuclease (~0.1 μg/ml) treatment also increases the survival of the streptomycin marker on wild-type cells, but it decreases the survival observed for the more UV-resistant cathomycin marker assayed on wild-type cells. Incubation with endonuclease at 0.4 μg of protein per ml results in a rapid, initial increase in activity for those markers reactivated with the smaller amount of endonuclease, and the increases are followed by decreases as the incubation proceeds for a longer time. The amount of inactivation obviously depends on the strain used for assay. It is greater for the wild-type than for DB112. Within experimental error, there are no effects on unirradiated DNA.

**DISCUSSION**

An endonuclease from *M. luteus* contains three activities: an endonuclease activity toward UV-irradiated native DNA that results in single-strand breaks near pyrimidine dimers but no excision of dimers (3), an activity that results in the increase of transforming ability of UV-irradiated *H. influenzae* DNA when assayed on cells unable to do excision in vivo (12), and an activity that results in the decrease in transforming ability for some markers when assayed on wild-type *H. influenzae*. The three activities have similar properties and we have not been able to separate them. Therefore we assume that they are all associated with the same protein molecules.

The largest biological reactivation is observed for assays carried out with strain DB112. This result implies that the endonuclease, acting on UV-irradiated DNA, supplies a function that is missing in these cells. This function is probably the first step in the excision process—the incision or formation of single-strand breaks near dimers. Presumably cells of strain DB112 can do the remaining steps in excision and repair of DNA. There is considerable evidence that pyrimidine dimers in transforming DNA are incorporated into the recipient genome (1, 12). However, we have not done the direct experiment to see whether dimers are excised from UV-irradiated DNA that has been treated with endonuclease and then integrated into the genome of DB112.

Although there is much evidence that pyrimidine dimers interfere with the replication and the transcription of DNA (6, 10, 14), it is clear that cells can grow and divide with dimers in their DNA (7, 14). The excision process, however, increases the probability of survival. Measurements in vivo of excision and of single-strand gaps associated with excision (12, 13) indicate that existing gaps are few in number during
FIG. 7. Comparison of the effects of endonuclease (~50-fold more active than that used in the experiments described in Fig. 1) on UV-irradiated transforming DNA. The markers and the strains used for assay are indicated. (a) Endonuclease at 0.1 μg of protein/ml, (b) endonuclease at 0.4 μg of protein/ml. The inset in (a) shows the degradation—measured by strand breaks—in a reaction containing ~10-fold higher concentration of photoproducets than in the transformation experiments.

excision. This result means that the processes of excision and of closing the gaps are temporally close to one another. When the gaps are not closed quickly, the DNA in vivo contains large numbers of single-strand breaks. Such breaks could upset the usually well-coordinated sequence of steps that result in repair. The DNA is subject to exonuclease attack and may be degraded before the final repair steps can be accomplished. Such an interpretation may be the proper one for some of the so-called "reckless" strains of E. coli (7). These strains probably can excise dimers (4), but this process results in massive DNA breakdown and very low survival after UV-irradiation.

Patrick and Rupert (9) observed that treatment of irradiated DNA with extracts of M. luteus led to inactivation only and that the UV-resistant markers were inactivated more than the sensitive ones. We suggest that the loss of activity of the UV-irradiated cathomycin marker upon treatment with endonuclease and assay on wild-type cells is the result of an upset in the usually well-coordinated sequence of steps of integration and repair. The UV-irradiated, endonuclease-treated DNA that goes into the cell has many strand breaks and it may lose activity on the introduction of further breaks by endogenous endonuclease or by exonuclease attack. After extensive endonuclease action on UV-irradiated DNA there is an activity loss observed when DB112 is the recipient cell. This
could be the result of (i) actions of in vivo nucleases on the large number of single-strand breaks, (ii) the failure to incorporate some of the smaller single-strand pieces into the recipient's genome, and (iii) the in vitro action of contaminating exonucleases present in amounts below our detection sensitivity (3). Despite the uncertainties in the above interpretation, it is clear (see Fig. 1 and 7) that the action of the UV-endonuclease on UV-irradiated DNA restores the transforming activity assayed on defective cells to approximately the level observed in wild-type cells.

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