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Genetics

Excision repair at individual bases of the *Escherichia coli lacI* gene: Relation to mutation hot spots and transcription coupling activity

(oligonucleotide-directed end-labeling/strand-specific repair/ultraviolet light mutagenesis)

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ABSTRACT **To determine whether base-to-base variations in the rate of excision repair influence the distribution of mutations, we have developed a method to measure UV photoproducts at individual nucleotides in the *Escherichia coli* chromosome. Specific gene fragments are 3′ end-labeled using a sequence-specific oligonucleotide to direct the site of labeling, and photoproducts are identified by enzymatic incision. On the nontranscribed strand of the *E. coli lacI* gene, the cyclobutane pyrimidine dimer frequency was 2- to 8-fold higher in chromosomal DNA than in a cloned DNA fragment. The chromosomal lesion frequency corresponded to the frequency of UV-induced mutations at mutation hot spots reported in the literature. Only 0–30% of cyclobutane dimers at various sites on this strand were excised in 20 min. In contrast, repair on the transcribed strand was 80–90% complete in 20 min. However, the transcribed strand contained an excision repair "slow spot" at the site of its single mutation hot spot: At this site, no repair occurred for the first 10 min, after which repair proceeded more slowly than typical of that strand. In an *mfd* strain, deficient in a factor that couples repair to transcription in cell extracts, the excision rate at individual nucleotides on the transcribed strand was minimal at most sites for at least 30 min. Wild-type *E. coli*'s bias for producing mutations at photoproducts on the nontranscribed strand, reported to require the *mfd* gene, therefore appears to be due to an excision repair system specific for the transcribed strand of chromosomal DNA.**

The pivotal role of DNA repair in preventing tumors is illustrated by the inherited disease xeroderma pigmentosum, in which an inability to repair UV photoproducts leads to elevated mutation frequencies and a 2000-fold increase in skin cancers (1). This effect is presumed to be due to reducing the frequency of the mutated oncogenes or tumor suppressor genes usually found in human tumors (2, 3). Not often considered is the extent to which base-to-base variations in excision repair might determine the location of mutations within these genes. Two candidates for such an effect are the nonrandom location of mutations at mutation hot spots and the bias for mutations at damage sites on the nontranscribed DNA strand.

Mutation hot spots are base pairs at which mutations occur much more frequently than at surrounding sites. Hot spots are evident in the p53 tumor suppressor gene in human colon, liver, and skin cancers; they are apparently due to 5-methylcytosine deamination, aflatoxin B_1 plus hepatitis B virus, and UVB (290–320 nm) wavelengths of sunlight, respectively (refs. 4–6; unpublished data). Similarly, inbred rodents often develop tumors having a particular oncogene activated at a particular site, the oncogene and site varying with the carcinogen used. For example, rat mammary tumors initiated by *N*-nitroso-*N*-methylurea carry mutations at the second G of codon 12 of the *HRAS* oncogene, even though the same mutation at the first G is also transforming (2).

A second type of specificity in the location of mutations is the bias for producing UV-induced mutations at base pairs where the UV photoproduct would have been formed on the nontranscribed DNA strand. This bias has been reported for genes in *Escherichia coli* and mammalian cells (7–10). It has recently been found that excision repair is particularly rapid on the transcribed strand of active genes (11, 12); this relation between excision and transcription appears to be due to a transcription-repair coupling factor, coded for in *E. coli* by the *mfd* gene (13, 14). The accompanying paper shows that the bias for producing UV mutations on the nontranscribed strand of the *E. coli lacI* gene is in fact due to the *mfd* gene (15).

Since the current methodology of gene- and strand-specific repair does not allow study of excision repair at single nucleotide resolution, we developed a method in which an oligonucleotide directs the end-labeling of a single gene in the bacterial chromosome. Using this technique, we examined the excision repair of cyclobutane pyrimidine dimers at the DNA sequence level in the *lacI* gene of *E. coli*.

MATERIALS AND METHODS

UV Irradiation and DNA Isolation. An overnight culture of *E. coli* K-12 strain GM1 [*araD(lac pro)*/F′ *lac pro*, I^q, L8] (from E. Eisenstadt, Harvard) was diluted 1:25 (to an absorbance of 0.1 at 600 nm) in Luria broth and grown until late lag phase (45 min). Some experiments used *uvrB5* or *mfd* derivatives of GM1 [from E. Eisenstadt and R. Schaaper (National Institute of Environmental Health Sciences)]. Cells were resuspended in medium 56 to an absorbance of 0.3. Fifteen milliliters of the suspension was spread in a 100-mm Petri dish and, while shaking, UV irradiated at a dose of 40 J/m^2 with a single 15 W General Electric germicidal lamp emitting principally 254-nm radiation. The dose rate was 1 J/m^2 per sec, measured with a UVX digital radiometer (Ultraviolet Products, San Gabriel, CA). Cell survival was 3% at this dose. The irradiated culture was then supplemented with 0.25% Casamino acids, 0.004% thiamine, and 0.4% glucose and incubated at 37°C for varying lengths of time. Portions of the culture were removed at regular intervals and genomic DNA was rapidly isolated. The bacterial cell pellet was suspended in 140 μl of Tris-EDTA, and 8 μl of 10% SDS and 2 μl of proteinase K (20 mg/ml) were added. The tubes were mixed thoroughly by inverting, incubated at 65°C for 5 min, and microwaved in a plastic weigh boat on ice for 30 sec. DNA was then isolated using cetyltrimethylammonium bromide (16), yielding ≈10 μg (3 × 10^9 copies of *lacI*). From UV irradiation to phenol extraction, all manipulations were carried out in dim yellow light to avoid removal of cyclobutane dimers by photoreactivation.

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Oligonucleotide-Directed End-Labeling. To measure excision repair at the DNA sequence level, we first developed an oligonucleotide-directed method of end-labeling a specific gene in the chromosome. The protocol for labeling the nontranscribed DNA strand is outlined in Fig. 1. An oligomer designed to be complementary to the 3′ end of the desired chromosomal fragment has six dTTPs on its 5′ end. This extension enables the chromosomal fragment to be extended with [^{32}P]dATP, using Taq DNA polymerase under stringent hybridization conditions. In practice, one chromosomal band in addition to the *lacI* band became labeled, a different extra band appearing with each choice of ^{32}P-labeled dNTP. The extra fragments appear to be hairpin structures, since they label even without an oligonucleotide primer.

For end-labeling, reaction mixtures contained 100 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 μg of restriction-digested genomic DNA, 250 ng of oligomer, 5 μCi of [^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq), and 5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus) in 100 μl. The samples were denatured at 94°C for 5 min and subjected to five cycles of denaturation, annealing, and extension in a Perkin–Elmer thermal cycler. The cycling conditions were 94°C, 1 min, and 72°C, 2 min, for *Apa* I–*Eco*RV-digested DNA and 94°C, 1 min, 55°C, 1 min, and 72°C, 2 min, for *Hin*cII–*Hae* III-digested DNA. Multiple cycles were used to maximize primer annealing and extension. To ensure that all DNA fragments were extended to full length, 2 μl of 10 mM unlabeled dATP was then added to bring the concentration of dATP above the K_m for Taq polymerase, and the reaction was continued for another five cycles. The reaction was stopped by adding 10 μl of 0.5 M EDTA; after phenol/chloroform extraction, the DNA was precipitated with ethanol.

For analyzing the *lacI* DNA region that contained most of the UV-induced nonsense mutation hot spots, base pairs 630–770 [numbered from the transcription start site (17)], the genomic DNA had been digested with *Apa* I and *Eco*RV, which leave 5′ overhanging and blunt ends, respectively. The resulting 240-base-pair (bp) fragment was then labeled at one of the 3′ ends using the oligonucleotide-directed end-labeling technique. The two oligomers used were, for the nontranscribed strand, 5′-TTTTTTATCCGCACCAACGCG-CAGCCCGGACTC-3′ and, for the transcribed strand, 5′-TTTTTTGGCCCATTAAGTTCTGTCTCGGCGCGT-3′. The *lacI* DNA fragment that contains most of the UV-induced missense mutation hot spots lies between base pairs 30–150. To analyze this region, the DNA had been digested with *Hin*cII and *Hae* III. The oligomers used were, for the nontranscribed strand, 5′-TTTTTTCCTGGTTCACCACG-CGGGAAACGG-3′ and, for the transcribed strand, 5′-TTT-TTTGACACCATCGAATGGCGCAAAACC-3′.

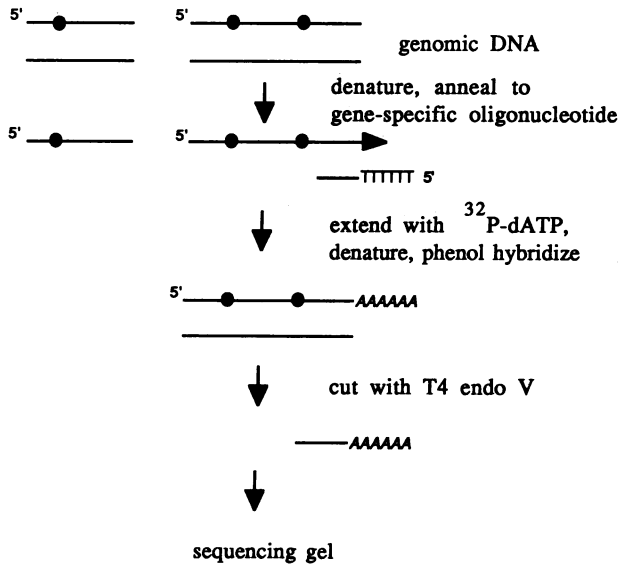

FIG. 1. Oligonucleotide-directed end-labeling. The oligonucleotide is used to 3′ end-label one restriction fragment of chromosomal DNA, by permitting Taq DNA polymerase to extend the fragment with radiolabeled dATP. The single-stranded DNA is then reannealed to permit the end-labeled fragment to be incised at sites of cyclobutane pyrimidine dimers (represented as dots). Fragments of various lengths correspond to various positions of cyclobutane dimers, and the frequency of dimers at a particular site determines the intensity of the corresponding gel band.

Analysis of Cyclobutane Pyrimidine Dimers. Photoproducts were identified by enzymatic incision. To facilitate cutting at cyclobutane pyrimidine dimer sites by T4 endonuclease V, which is most efficient on double-stranded DNA, the labeled *lacI* fragment was first renatured using the phenol/NaSCN-enhanced reassociation technique (18). A 2-hr hybridization at 0.1 mg/ml achieves a C_0t of ≈10,000 $C_0t_{1/2}$. After removing the phenol by chloroform extraction, the double-stranded *lacI* fragment (with only one strand labeled) was purified on a 5% polyacrylamide native gel. Appearance of all label in the double-strand band indicated that reannealing had been complete. After purification, the DNA was incised at sites of cyclobutane pyrimidine dimers with T4 endonuclease V as described (19). At the low UV doses used, >80% of the fragments have no dimers and are uncut. DNA fragments were separated on an 8% denaturing acrylamide gel, alongside standards containing known numbers of cpm. Approximately equal numbers of cpm were loaded in each lane. Untreated DNA migrated as a single band, indicating that all fragments had been completely extended during radiolabeling. Sites of dimers appear as gel bands, which were located and quantitated by scanning the autoradiogram using an LKB Ultroscan XL laser densitometer (Pharmacia). The percentage of fragments incised at a site was calculated as the ratio of radioactivity in the corresponding band to the total radioactivity loaded. The correction for multiple photoproducts in the same fragment (19) was found to be <10% and was omitted.

RESULTS

Initial Photoproduct Frequency. Cyclobutane pyrimidine dimers join adjacent pyrimidines. The initial distribution of these photoproducts after UV irradiation can be seen in the 0-min lanes of Fig. 2 *A–C*. Gel bands indicate the positions of cyclobutane dimers, and the intensity of a band indicates the frequency of photoproducts at that site. The frequency of cyclobutane pyrimidine dimers in the chromosomal *lacI* gene immediately after UV irradiation has been quantitated in Table 1 ("0 min" column). Allowing for dosimetry differences, the chromosomal frequency of TT dimers on the transcribed strand was equal to that expected by extrapolation from measurements made on a cloned *lacI* fragment at a 12-fold higher dose (20). However, the frequency of TC dimers was 2- to 4-fold higher in chromosomal DNA than in the cloned gene. Moreover, on the nontranscribed strand the frequency of TT and TC dimers was 2- to 8-fold higher in the chromosome than expected from the earlier results. The largest difference, and the most variable, was at site Am23, which ranged from 5- to 11-fold higher in different experiments.

Photoproduct Excision Rates. If DNA replication occurred during the post-UV incubation, the resulting dilution of photoproducts could be mistaken for repair. Therefore, we first measured the replication rate of the *lacI* gene after irradiation by isolating genomic DNA from equal volumes of the culture at different time periods after UV irradiation. Samples were restriction digested, Southern blotted, and probed with a cloned *lacI* probe. The number of copies of *lacI* DNA was constant for at least 1 hr after UV irradiation, indicating that there was no DNA replication (not shown).

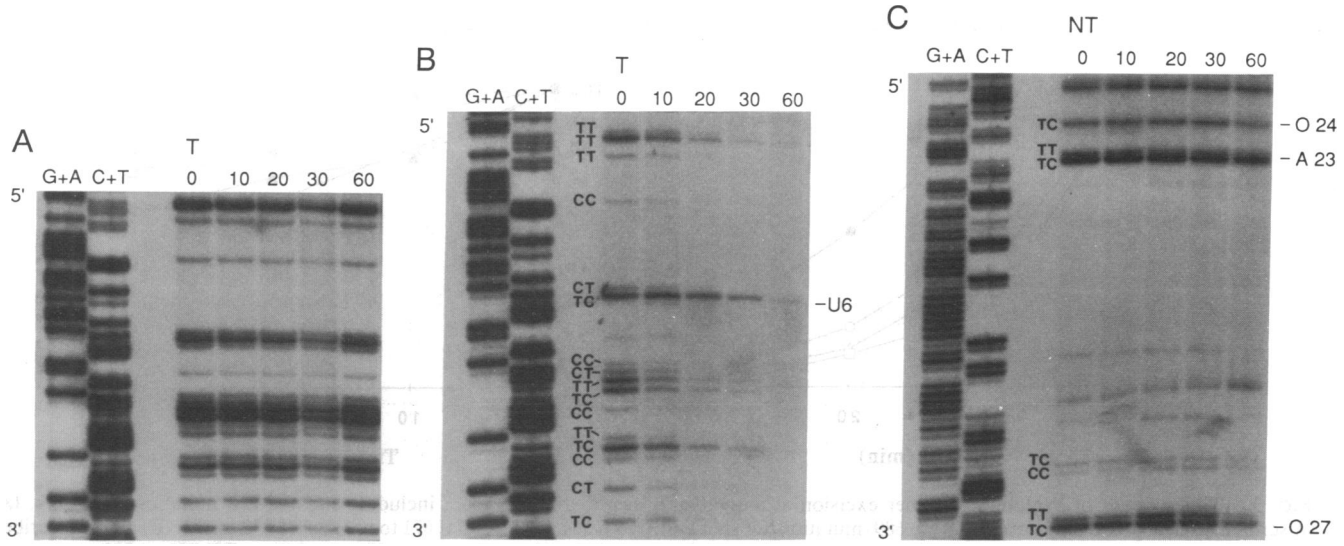

FIG. 2. Excision of cyclobutane pyrimidine dimers at the DNA sequence level in the *lacI* gene. Cells were irradiated with 40 J/m^2 254-nm UV light and incubated for 0–120 min, and the DNA was extracted for analysis of cyclobutane dimers in the region of base pairs 630–770. G+A and C+T, Maxam–Gilbert sequencing reactions. With 3′ end-labeled DNA, cyclobutane dimer bands migrate one or two bases slower than the 5′ member of the dipyrimidine pair. (*A*) Transcribed strand (T) of a *uvrB5* repair-deficient strain. (*B*) Transcribed strand (T) of a repair-proficient strain. The excision-repair slow spot at site U6 and other dipyrimidine sites are noted. (*C*) Nontranscribed strand (NT) of the repair-proficient strain.

So that our excision repair measurements would be comparable to previous measurements of the UV mutation spectrum (21–23), we used the same GM1 strain background and irradiated to the same survival level. In these strains, the *lacI* gene is on the F′ factor and the *lacI* promoter contains an up-promoter mutation, I^q, that results in 10-fold higher levels of Lac repressor than normal (24).

If cyclobutane dimers are repaired at a particular site, the intensity of the corresponding gel band will decrease compared to the intensity at 0 min. The intensity of the uncut band

Table 1. Repair of cyclobutane pyrimidine dimers at individual bases of the *E. coli lacI* gene

Strand, bp	Nonsense site (Muts)	Sequence (5′ → 3′)	% cyclobutane dimers*			
			0 min	10 min	20 min	30 min
Transcribed						
650 to 651		ATTG	0.12	0.05	0.005	0
654 to 655		ATTTG	0.29	0.08	0.03	0
655 to 656		ATTTG	0.14	0.04	0.02	0
664 to 665		ATCG	0.11	0.06	0.014	0
671 to 672		GTTCC	0.36	0.14	0.05	0.01
672 to 673		GTTCC	0.09	0.02	0.01	0
688 to 689	U6 (39)	ACTCCA	0.31	0.28	0.16	0.06
708 to 709		GTTG	0.04	0.01	0.002	0
711 to 712		GTTTG	0.41	0.14	0.03	0.01
720 to 721		ATTTG	0.27	0.11	0.02	0.01
721 to 722		ATTTG	0.19	0.07	0.01	0
727 to 728		ATTCA	0.51	0.32	0.08	0.03
728 to 729		ATTCA	0.22	0.08	0.04	0.01
731 to 732		CCTCA	0.68	0.33	0.14	0.03
Nontranscribed						
74 to 75		TCTCTT	0.58	0.58	0.58	0.58
79 to 80		ATCA	0.55	0.50	0.41	0.30
89 to 90		TTTCCC	1.09	1.01	0.84	0.58
652 to 653	Oc24 (43)	ATCA	0.32	0.31	0.25	0.25
657 to 658		ATTCA	0.36	0.33	0.27	0.22
658 to 659	Am23 (80)	ATTCG	0.67	0.65	0.53	0.49
706 to 707	Oc27 (60)	TTTCA	0.73	0.67	0.56	0.53
738 to 739		ATCG	0.19	0.15	0.14	0.13
741 to 742		GTTCCC	0.18	0.16	0.13	0.09
742 to 743		GTTCCC	0.81	0.71	0.59	0.49
759 to 760		GTTG	0.05	0.04	0.04	0.03
769 to 770	Am26 (2)	ATCA	0.08	0.06	0.05	0.05

Muts, normalized number of mutations reported in refs. 20 and 21; Am, amber; Oc, ochre; U, umber. Bases underlined are those involved in the cyclobutane dimer measured. Dimer frequencies are the average of three independent experiments, except base pairs 74–90 were measured once. Variation between experiments was ≈20%. Base numbering is from ref. 17.
*After UV irradiation.

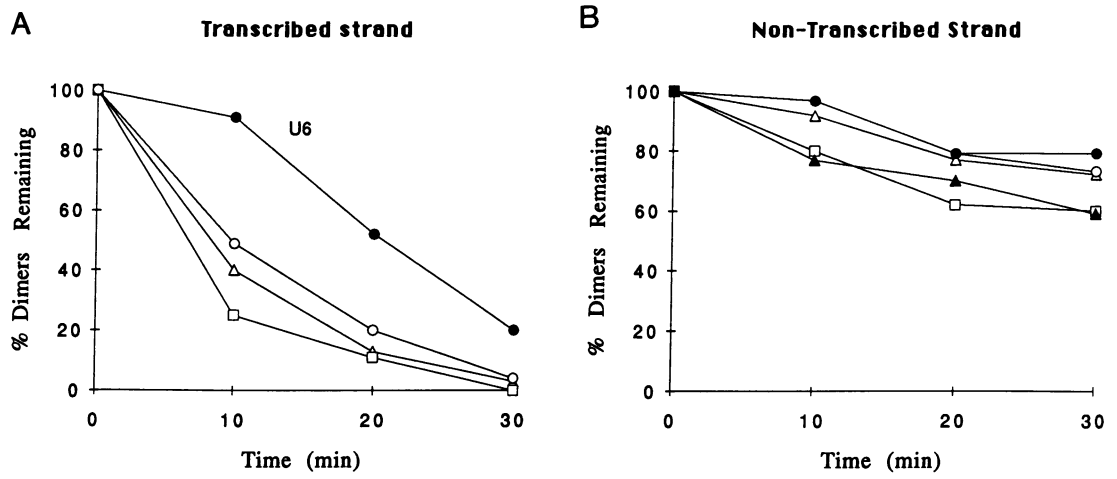

FIG. 3. Time course of cyclobutane dimer excision at individual bases of the *lacI* gene, including sites of UV mutation hot spots. (*A*) Transcribed strand. ●, TC dinucleotide 688 to 689 (mutation hot spot U6); ○, TC 731 to 732; △, TC 671 to 672; □, TT 672 to 673. (*B*) Nontranscribed strand. ●, TC 652 to 653 (Oc24); ○, TC 658 to 659 (Am23); △, TC 706 to 707 (Oc27); □, TC 769 to 770 (Am26); ▲, TT 759 to 760.

(not visible in the figures) will be greater than at 0 min. In the *uvr*$^-$ strain, repair of cyclobutane dimers in *lacI* was absent at all sites. The transcribed strand is shown in Fig. 2*A*. In contrast, the *uvr*$^+$ strain efficiently removed dimers from the transcribed strand (Fig. 2*B*). Repair was almost complete at most of the dimer sites in the transcribed strand within 20 min after irradiation. An exception was dinucleotide 688 to 689, the site of nonsense mutation hot spot U6 (Fig. 2*B*). At this site, no repair occurred for the first 10 min, after which repair proceeded more slowly than typical of that strand. Quantitation revealed that the greatest repair differences between sites occurred in the first 10 min (Fig. 3*A*) and that the U6 site was only 50% repaired by 20 min (Table 1). Dimers at TT were repaired more quickly than at TC.

The nontranscribed strand of *lacI* was repaired much more slowly than the transcribed strand (Figs. 2*C* and 3*B*). Repair at the Am23, Oc24, and Oc27 nonsense mutation sites was particularly slow during the first 10 min compared to other sites on the same strand (Fig. 3*B*). Similarly, no repair was detected on the nontranscribed strand at base pair 75 (Table 1); this site is the strongest missense mutation hot spot identified in the gene (23).

Transcription-Repair Coupling. In contrast to the rapid excision of cyclobutane dimers from the transcribed strand of chromosomal DNA in the wild-type strain, excision in the *mfd* strain was minimal at most sites for at least 30 min after UV irradiation (Fig. 4). These poorly repaired sites include base pair 31, shown in the accompanying paper to be the site of a pronounced UV mutation hot spot in the *mfd* strain (15).

DISCUSSION

Oligonucleotide-Directed End-Labeling. In principle, end-labeling avoids a problem inherent to ligation-mediated PCR (25) or primer extension methods, in which a frequent photoproduct such as a cyclobutane dimer will prevent observing a less frequent photoproduct, such as a (6-4) photoproduct, on the same DNA fragment. It also avoids site-to-site variations in ligation efficiency. The background from nonspecific reannealing and shear events was low enough to permit physiological UV doses to be used. The technique also provides a rapid substitute for Southern blotting.

Initial Photoproduct Frequency and Mutation Hot Spots on the Nontranscribed Strand. Photoproduct frequencies at many sites were found to be 2- to 8-fold greater in chromosomal DNA than in a cloned gene; the reason is unknown. Since the *lacI* gene was highly active, because of the I^q up-promoter mutation, the difference may relate to the 2- to 6-fold differences reported between double-stranded and melted DNA (26). If so, the efficiency of cyclobutane dimer induction in an inducible gene, such as *lacZ*, should vary with the extent of induction.

The fact that cyclobutane dimers at site Am23 were induced much more frequently in chromosomal DNA than in the cloned gene, relative to surrounding sites, alters the relation between mutations and lesions reported previously (20). In that study, the frequency of nonsense mutations was linearly related to the frequency of cyclobutane dimers and (6-4) photoproducts in the cloned gene, with the exception of site Am23. Mutations at Am23, the most frequent nonsense mutation hot spot, were 3-fold more frequent than could be accounted for by the lesion frequency. In contrast, the relative order of dimer frequencies *in vivo* was Am23 ≈ Oc27 > Oc24 >> Am26 (Table 1). Therefore, in this region of the gene the frequency of nonsense mutations on the nontranscribed strand reflects the *in vivo* lesion frequency. This correspondence does not require that the cyclobutane dimer is itself the mutagenic photoproduct, because other photoproducts not measured could be as frequent at these sites.

DNA Excision Repair and Mutation Hot Spots on the Transcribed Strand. The oligonucleotide-directed end-labeling procedure enabled observation of strand-specific repair at

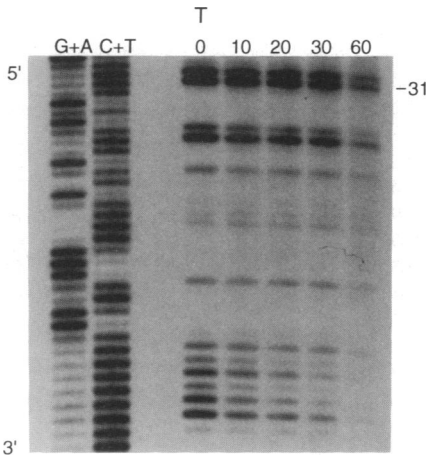

FIG. 4. Excision of cyclobutane dimers on the transcribed strand (T) of an *mfd* strain, defective in the transcription-repair coupling factor. The region shown spans base pairs 2–38. Excision is minimal at most sites. The site of the strong mutation hot spot in the *mfd* strain, base pair 31, is among those poorly repaired.

individual nucleotide resolution; in contrast, the standard Southern blot methodology averages repair rates over several kilobases. As anticipated from observations at the level of the entire gene, repair rates on the transcribed strand exceeded those on the nontranscribed strand (Figs. 2 and 3). A surprising feature of the transcribed strand was the presence of a site, dinucleotide 688 to 689, at which excision of cyclobutane dimers was delayed for 10 min before proceeding more slowly than at surrounding sites (Figs. 2*B* and 3*A*). A low-resolution examination of a larger region of the *lacI* gene, using a low-percentage denaturing acrylamide gel, showed two or three similar sites in the gene (not shown). We call these sites "excision repair slow spots." Since cyclobutane dimers and (6-4) photoproducts are repaired by the *uvrABC* excinuclease (27, 28), these sites may be slow spots for (6-4) photoproducts as well. The basis for the excision repair slow spots is unknown. Since dinucleotide 688 to 689 lies in a quasi-palindromic DNA sequence with the potential to form a hairpin loop (17), it may be that DNA secondary structures delay the onset of lesion excision within very localized regions. For example, UvrABC excinuclease has been shown to require negative DNA supercoiling for incision of psoralen crosslinks (29).

In the *lacI* gene, UV induces 9 nonsense and 7 missense mutation hot spots, but for only one of these base pairs do the adjacent pyrimidines lie on the transcribed strand (21–23). Fig. 2*B* shows that this hot spot, nonsense site U6, lies at the site of the excision repair slow spot. This result suggests that excision repair rate influences the location of mutation hot spots on the transcribed strand, by removing lesions at other sites more rapidly. For the remaining 15 mutation hot spots the lesions responsible lie on the nontranscribed strand, where there is little repair.

Whereas the excision repair system has long been known to reduce the overall frequency of mutations in *E. coli* (13), the correspondence between UV mutation hot spots and (*i*) the excision repair slow spot and (*ii*) the difference in excision rate between strands suggests that site-to-site variations in the *rate* of excision can profoundly affect mutation frequency, even if excision is eventually complete. Many of the mutations that occur in wild-type cells become irreversible during the first 20 min after UV irradiation (30, 31). Since the inducible, error-prone, SOS system is required for UV mutagenesis (32, 33), a short delay in repair may result in substantially more photoproducts being present as SOS is being induced.

Transcription-Repair Coupling. The *mfd* mutant is known to excise cyclobutane dimers from the overall genome more slowly than does the wild type (34). The absence of observable excision of most individual cyclobutane dimers on the transcribed strand of *lacI* in the *mfd* strain (Fig. 4) indicates that the Mfd protein is required for repair on that strand. Since the UvrABC excinuclease is already known to be required for excision of UV photoproducts (27, 28), the Uvr and Mfd proteins evidently cooperate. Particular models for the molecular mechanism of transcription-repair coupling, and for the generation of strand bias in UV-induced mutations, are discussed in the accompanying paper (15).

That paper also reports a 300-fold increase in mutations at base pair 31 in the *mfd* strain, with the mutating pyrimidine located on the transcribed strand. The authors noted that this result could indicate either that base pair 31 was particularly dependent on repair by the Mfd protein or that this site was atypical only in being a phenotypically scorable site that could be mutated by the most frequent UV-induced event, a C → T substitution at a TC sequence. The uniformity of slow cyclobutane dimer excision on the transcribed strand of the *mfd* strain suggests that the latter possibility is the case.

DNA excision repair in the *E. coli* chromosome therefore appears to vary from strand to strand and from base to base. There are also some nucleotides at which the production of UV damage in the chromosomal *lacI* gene differs from that in the cloned gene. These variations correspond to similar variations in the location of mutations.

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