Preferential repair of cyclobutane pyrimidine dimers in the transcribed strand of a gene in yeast chromosomes and plasmids is dependent on transcription

(transcription-associated repair/UV photoproducts)

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ABSTRACT   While preferential repair of the transcribed strands within active genes has been demonstrated in organisms as diverse as humans and *Escherichia coli*, it has not previously been shown to occur in chromosomal genes in the yeast *Saccharomyces cerevisiae*. We found that repair of cyclobutane pyrimidine dimers in the transcribed strand of the expressed *RPB2* gene in the chromosome of a repair-proficient strain is much more rapid than that in the nontranscribed strand. Furthermore, a copy of the *RPB2* gene borne on a centromeric *ARS1* plasmid showed the same strand bias in repair. To investigate the relation of this strand bias to transcription, we studied repair in a yeast strain with the temperature-sensitive mutation, *rpb1-1*, in the largest subunit of RNA polymerase II. When exponentially growing *rpb1-1* cells are shifted to the nonpermissive temperature, they rapidly cease mRNA synthesis. At the permissive temperature, both *rpb1-1* and the wild-type, parental cells exhibited rapid, proficient repair in the transcribed strand of chromosomal and plasmid-borne copies of the *RPB2* gene. At the nonpermissive temperature, the rate of repair in the transcribed strand in *rpb1-1* cells was reduced to that in the nontranscribed strand. These findings establish the dependence of strand bias in repair on transcription by RNA polymerase II in the chromosomes and in plasmids, and they validate the use of plasmids for analysis of the relation of repair to transcription in yeast.

Both eukaryotes and prokaryotes carry out excision repair of DNA damage after exposure to UV light (1). The two major classes of lesions produced are 5-5, 6-6 *cis-syn* cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts, both of which are removed from the DNA. Furthermore, CPD removal has been shown in mammalian cells to be faster in transcriptionally active genes when compared to the genome overall (for recent reviews, see refs. 2 and 3) and more rapid in the transcribed strand in expressed genes than in the nontranscribed strand (4). This preferential repair of the transcribed strand in expressed genes has also been shown for the *lac* genes in the *Escherichia coli* chromosome (5) and for the *uvrC* gene *in vitro* (6). Thus, it seems likely that preferential DNA repair of active genes is a conserved pathway for nucleotide excision repair in both prokaryotes and eukaryotes. However, preferential DNA repair of the transcribed strand of an active chromosomal gene has not yet been shown in the unicellular eukaryote *Saccharomyces cerevisiae*.

Recent reports of transcription-associated nucleotide excision repair in yeast are seemingly inconsistent. Studies of repair in unique chromosomal DNA sequences did not show a strand bias in repair (7–9). In contrast, Smerdon and Thoma and colleague (10, 11) reported that excision repair of CPDs in a plasmid was rapid for transcribed strands, while nontranscribed sequences were slowly repaired. To resolve these differences we have compared the repair rates in the same expressed sequence in a plasmid and in the chromosome.

Using haploid yeast strains containing two copies of the *RPB2* gene, encoding the second largest subunit of RNA polymerase II, one copy on an *ARS1-CEN4* plasmid and the other in a chromosome, we have demonstrated that preferential repair of the transcribed DNA strand in a specific gene occurs in both locations. Furthermore, this preferential repair is rapid and dependent on transcription by RNA polymerase II (pol II). This conclusion was based on experiments with a yeast mutant carrying a temperature-sensitive mutation in the largest subunit of RNA pol II, *RPB1*. By comparing the rates of repair in the mutant at the permissive and nonpermissive temperatures, we have established that transcription is required for strand-specific excision repair of CPDs in this gene, both in the chromosome and in a plasmid.

MATERIAL AND METHODS

Media, Plasmids, and Strains. YPD medium is 1% yeast extract/2% Bacto-peptone (Difco)/2% glucose (12). Synthetic glucose medium (SD) is 2% glucose/0.67% Bacto-yeast nitrogen base without vitamins (Difco) supplemented with amino acids and bases except for uracil (12). Agar (1.5%) was added to medium for plates. Yeast RNA pol II mutant strain Y260 (*MATα rpb1-1 ura3-52*) and its parent DB1033 (*MATα ura3-52*) were both gifts from R. Young (Massachusetts Institute of Technology) (13). Plasmid pRP212, also a gift from R. Young, is a 13.6-kilobase (kb) *ARS1-CEN4* plasmid containing the selectable marker *URA3* and the gene *RPB2*, encoding the second largest subunit of RNA pol II (14). Transformation of Y260 and DB1033 with pRP212 generated strains YSH11 and YSH10, respectively. Plasmid pKS212 was constructed by cloning the internal 1.0-kb *Eco*RI/*Xho* I from RPB2 into the multiple cloning site of Bluescript pKS+ (Stratagene). Strand-specific RNA probes for *RPB2* were synthesized by cleaving pKS212 with *Xho* I or *Eco*RI and incubating the linearized plasmid with rNTPs and T7 RNA polymerase or T3 RNA polymerase, respectively, under conditions recommended by the manufacturer.

Growth and UV Irradiation of Yeast Cells. Both YSH11 and YSH10 were grown in minimal SD medium supplemented with the appropriate amino acids at 24°C until cells reached $A_{600} \approx 2.5$ (3.0 × 10⁷ cells per ml), at which time half of each culture was kept at the permissive temperature (24°C) and the other half was shifted to the nonpermissive temperature (36°C) and then incubated for 30–150 min to ensure arrest of RNA pol II transcription. Cells were collected by centrifu-

Abbreviations: CPD, cyclobutane pyrimidine dimer; RNA pol II, RNA polymerase II.
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gation or filtration and resuspended in ice-cold phosphate-buffered saline at 1×10^7 cells per ml. The cell suspensions were transferred to Pyrex dishes ($25 \times 15 \times 4$ cm) such that the depth of the suspension was ≈ 0.2 cm to ensure a uniform UV dose to all cells. Shaking cell suspensions were irradiated with predominantly 254-nm UV light (30 J·m⁻²) at 0.33 J·m⁻²·s⁻¹ using a Westinghouse IL782-30 germicidal lamp. After irradiation, the cells were collected by centrifugation or filtration and either lysed immediately or resuspended in their original growth medium at 24°C or 36°C. Cells were incubated for various times to allow DNA repair and then lysed. All manipulations were performed under yellow light to prevent photoreactivation.

Transformation of Yeast. Yeast were transformed by the lithium acetate method (15) with modifications (16). Transformants were isolated by growth on selective medium.

Isolation of Yeast DNA. Cells were lysed with Zymolyase 100T as described by Sherman *et al.* (12) with modifications from Smerdon and Thoma (10). Cesium chloride was added to the lysates such that the final density was 1.55 g/ml. Ethidium bromide was added to a final concentration of 400 μg/ml. DNA was separated from RNA, protein, and cellular debris by centrifugation to equilibrium as described (12). Chromosomal and plasmid DNA were collected together or separately from the gradient with a syringe. Ethidium bromide was removed by several extractions with 1-butanol and the DNA was precipitated directly from the CsCl solution by the addition of 3 vol of water and 8 vol of ethanol (17). Purified DNA was resuspended in 10 mM Tris·HCl, pH 7.5/1 mM EDTA and stored at 4°C.

Analysis of Frequency of CPDs. The incidence of CPDs in a particular restriction fragment was determined by methods developed in this laboratory (4, 18). Briefly, 1 μg of purified and restricted DNA in 10 mM Tris·HCl, pH 7.5/0.1 M NaCl/10 mM EDTA/1 mg of bovine serum albumin per ml was digested with T4 endonuclease V in 40 μl for 30 min at 37°C. The specific activity of the T4 endonuclease V preparation (0.71 mg/ml) was 5–8 × 10¹¹ nicks per min per μl on 250 ng of pSV2gpt irradiated with 32 J/m²·s (≈1 dimer per plasmid) and ≈1 × 10⁸ nicks per min per μl on unirradiated DNA (Ann Ganesan and P.C.H., unpublished data). Reactions were stopped by the addition of 10 μl of 12.5% Ficoll/5 mM EDTA/0.125% bromophenol blue/0.25 M NaOH. Samples were immediately loaded into 1.0% alkaline agarose gels under 30 mM NaOH/1 mM EDTA and electrophoresed at 1.7 V/cm overnight with recirculating buffer. DNA was transferred to Hybond N⁺ (Amersham). Membranes were prehy-

bridized for at least 2 hr and then hybridized with strand-specific RNA probes made from pKS212.

RESULTS

The Transcribed Strand of RPB2 Is Preferentially Repaired Both in the Chromosome and in a Plasmid. We compared the removal of CPDs from the transcribed strand and the non-transcribed strand of the *RPB2* gene. The repair-proficient yeast strains DB1033 and Y260 were transformed with pRP212, a selectable *ARS1-CEN4* plasmid containing the *RPB2* gene. Y260 is an *rpb1-1* mutant derived from DB1033 (13). The resulting transformants YSH10 and YSH11 contain two expressible copies of *RPB2*, one in the plasmid and the other in the chromosome. Cloning of the *RPB2* gene into pRP212 resulted in loss of a *Pvu* II recognition site upstream of the *RPB2* promoter. Digestion of plasmid and chromosomal DNA with restriction endonucleases *Pvu* I and *Pvu* II generates a 5.8-kb restriction fragment from the plasmid-borne copy of the *RPB2* gene and a 5.2-kb restriction fragment from the chromosomal copy (Fig. 1). After treatment with the CPD-specific enzyme T4 endonuclease V, samples were electrophoresed along with untreated control samples in alkaline agarose gels, transferred to membranes, and hybridized with strand-specific RNA probes. In this way, we can compare directly the CPD level and repair of the gene in a plasmid and in the chromosome in the same experiment.
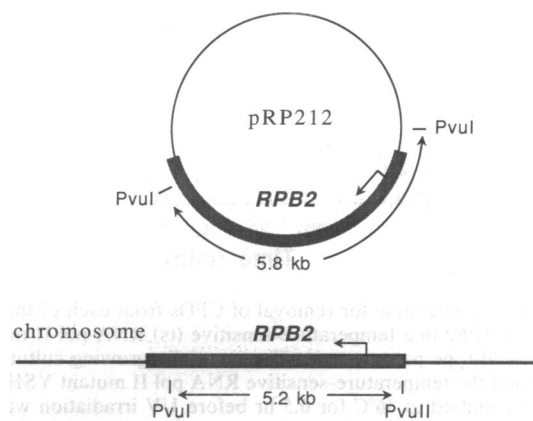

FIG. 1. Restriction fragments containing the *RPB2* gene. Digestion of plasmid and chromosomal DNA with restriction endonucleases *Pvu* I and *Pvu* II results in a 5.2-kb restriction fragment from the chromosomal copy of the *RPB2* gene and a 5.8-kb restriction fragment from the plasmid.
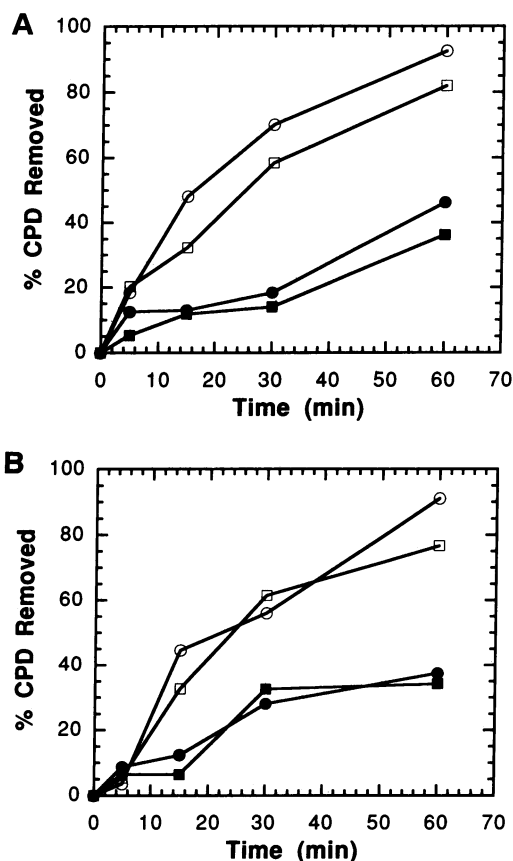

FIG. 2. Time course for removal of CPDs from the transcribed and nontranscribed strands of *RPB2* in the chromosome and plasmid. Exponentially growing cultures of YSH10 (*A*) and YSH11 (*B*) at 24°C were UV irradiated (30 J/m²) and incubated in growth medium for the times indicated. Repair was determined from the measured incidences of CPDs in the restriction fragments. □, Transcribed strand, chromosome; ■, nontranscribed strand, chromosome; ○, transcribed strand, plasmid; ●, nontranscribed strand, plasmid.

We examined the time course of repair of the individual strands of *RPB2* at 24°C in exponentially growing cultures of strains YSH10 and YSH11, irradiated with 30 J/m² (Fig. 2). In both strains, the transcribed strand was repaired more rapidly than the nontranscribed strand. This was true for the chromosomal and plasmid-borne copies of *RPB2*. In both strains, the rate of repair of the plasmid-borne copy of *RPB2* was similar to that of the chromosomal copy.

Preferential Repair Requires RNA Pol II Transcription. The association of strand bias in repair with transcription has been well documented for mammalian and bacterial cells (2, 3). For example, the RNA pol II inhibitor α-amanitin has been shown to inhibit the strand bias in repair of transcribed genes in rodent (19) and human cells (20, 21). Yeast strains containing the mutant *rpb1-1* allele of the gene encoding the largest subunit of RNA polymerase II quickly and selectively cease RNA pol II transcription when shifted to 36°C (13). This temperature-sensitive mutation provides a convenient method to investigate the role of transcription in preferential repair in yeast.

We compared the rates of repair of the *RPB2* gene in the chromosome and in a plasmid in YSH10 and YSH11 at the nonpermissive temperature. Exponentially growing cultures of YSH10 and YSH11 at 24°C were shifted to 36°C for 0.5–2.5 hr before UV irradiation with 30 J/m². Cells were returned to their original medium and incubated at 36°C for 0–60 min. An autoradiograph of a membrane from this experiment, hybridized with an RNA probe for the transcribed strand, is shown in Fig. 3. The initial CPD frequencies for both the transcribed and nontranscribed strands of *RPB2* in YSH11 were similar to those observed for YSH10 (data not shown). For the

chromosomal copy, the transcribed strand of *RPB2* was as efficiently repaired at 36°C in YSH10 (Fig. 4A) as it was at 24°C. Within 5 min of irradiation, 35% of the CPDs were removed from the transcribed strand while <10% were removed from the nontranscribed strand. In contrast to the results obtained with YSH10, YSH11 cells exhibited no preferential removal of CPDs from the transcribed strand when incubated at the nonpermissive temperature (Fig. 4A); the repair of the transcribed strand was reduced to the level observed for the nontranscribed strand in YSH10 and YSH11 (<40% removal of CPDs by 30 min).

The rates of removal of CPDs from the transcribed strand of the plasmid-borne copy of *RPB2* were the same as those for the chromosomal copies in both YSH10 and YSH11 (Fig. 4B). Thus, the strand-specific repair in both the chromosome and in the plasmid is dependent on RNA pol II transcription.

DISCUSSION

We measured the removal of UV-induced CPDs from each of the two strands of the expressed *RPB2* gene of yeast carried on a plasmid and in the chromosome. The transcribed *RPB2* gene was chosen for study because of its size and not because its protein product is involved in transcription. We found that the rates and extent of repair were similar for both locations of the gene (Fig. 2). Repair was very rapid in the transcribed
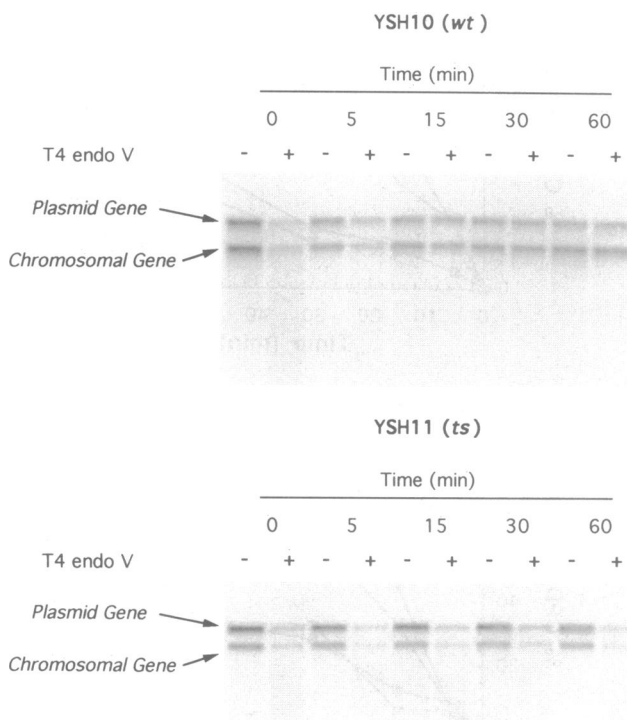

YSH10 (*wt*)

FIG. 3. Preferential removal of CPDs in a transcribed gene is dependent on RNA pol II transcription. Exponentially growing cultures of YSH10 and YSH11 (containing a temperature-sensitive RNA pol II mutation) at 24°C were shifted to 36°C for 0.5 hr before UV irradiation with 30 J/m². Postirradiation incubation in growth medium was at 36°C for the times indicated. The autoradiograph shown is of a membrane hybridized with RNA probes specific for the transcribed strand of *RPB2*. wt, Wild type; ts, temperature sensitive.
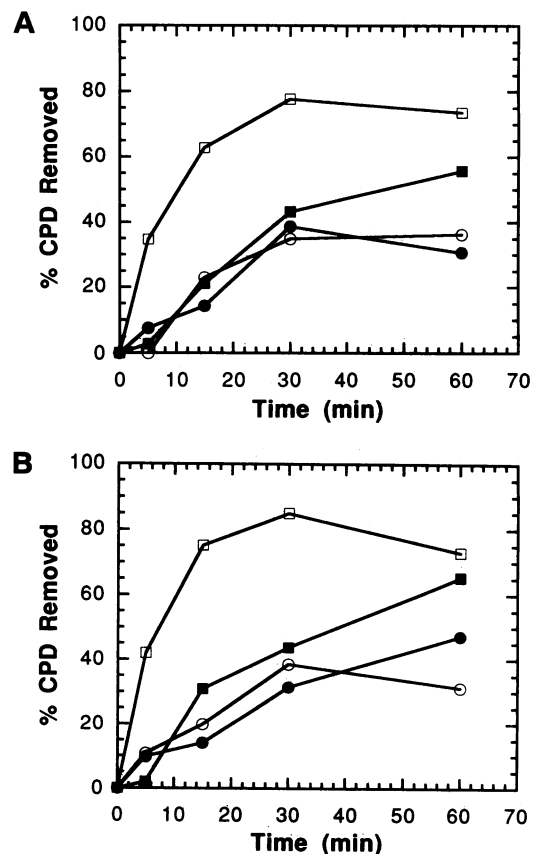

FIG. 4. Time course for removal of CPDs from each of the two strands of *RPB2* in a temperature-sensitive (ts) RNA pol II mutant and the wild-type parent strain. Exponentially growing cultures of YSH10 and the temperature-sensitive RNA pol II mutant YSH11 at 24°C were shifted to 36°C for 0.5 hr before UV irradiation with 30 J/m². After irradiation, the cultures were incubated in growth medium at 36°C for the times indicated. Repair was determined from the measured incidences of CPDs in the restriction fragments from the chromosome (*A*) and the plasmid (*B*). Transcribed strand, YSH10 (□); nontranscribed strand, YSH10 (■); transcribed strand, YSH11(*ts*) (○); nontranscribed strand, YSH11(*ts*) (●).

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strand, with most of the CPDs being removed within 30 min, while repair of the nontranscribed strand was much slower and similar to that of the overall genome as reported by others (11, 22). In addition, we were able to show that the rapid repair of the transcribed strand was dependent on transcription by RNA pol II. A strain carrying a temperature-sensitive mutation in the largest subunit of RNA polymerase II did not exhibit preferential removal of CPDs from the transcribed strand of the *RPB2* gene at the nonpermissive temperature (Figs. 3 and 4).

When we initiated our study, we had assumed that both copies of the *RPB2* gene would be transcribed when present in the same cell. Certainly, the plasmid-borne copy was thought to be transcribed since it contains the chromosomal promoter of *RPB2* as well as a bacterial sequence in the vector that acts as a strong RNA pol II promoter in both yeast and mammalian cells (23). This bacterial sequence is in the same orientation as the *RPB2* promoter in pRP212. The dependence on temperature of the rate of removal of CPDs from the transcribed strand of both copies of *RPB2* in YSH11 suggests that both copies are indeed expressed.

Recent studies from separate laboratories concerning transcription-associated nucleotide excision repair within yeast genes in a plasmid or in the chromosome are seemingly inconsistent. Terleth *et al.* (7, 8) compared the rates of excision repair of CPDs in a chromosomal sequence from the transcriptionally active *MATα* locus and the same sequence from the transcriptionally repressed *HMLα* locus. They observed efficient repair of the *MATα* sequence but little or no repair in the *HMLα* locus. However, they subsequently reported that in a mutant containing a promoter deletion for the two divergent transcripts of *MATα*, the nontranscribed sequence was as efficiently repaired as the intact *MATα* (9). Thus, something other than the transcriptional activity of *MATα*, perhaps chromatin structure, is the decisive factor in determining its preferential gene-specific repair over *HMLα*. This is contradictory to the results obtained for the excision repair of CPDs in a yeast plasmid (10). Using indirect end-labeling and T4 endonuclease V, Smerdon and Thoma (10) investigated the excision repair of CPDs from both transcribed and nontranscribed genetic elements (*URA3*, *ARS1*, nucleosome binding sites) throughout the 2.6-kb multicopy *ARS1* plasmid, TRURAP. The rates of repair varied widely throughout the plasmid. The transcribed strand of the expressed *URA3* gene was efficiently repaired, while the nontranscribed strand of *URA3* and both strands of the *ARS1* region were poorly repaired. These latter results resemble those observed in mammalian cells and *E. coli*, for which it has been concluded that strand bias in repair of genes is associated with transcription. Indeed, regions of the TRURAP plasmid, which showed proficient repair of presumed nontranscribed sequences, were later shown to be transcribed (11).

Several differences between the experimental systems and approaches used by these investigators may explain their dissimilar observations. First, Terleth *et al.* (7, 8) determined the rates of repair of the repressed *HMLα* locus, which possesses a chromatin structure quite unlike that of the yeast genome overall (24). Hence, *HMLα* may not be representative of unexpressed genes, only representative of transcriptionally repressed regions of the genome. Second, while the chromatin structure of the transcribed *MATα* locus is similar to that of *HMLα*, they are not identical (25). *MATα* is more sensitive to DNase I cleavage and might be more accessible to repair enzymes. Third, the transcribed *MATα* possesses two divergent transcripts together spanning only ≈1.7 kb of the 4.0- to 4.4-kb restriction fragment in which repair was investigated. In contrast, Smerdon and colleagues (11) studied excision repair of a plasmid folded into chromatin and found repair rates comparable to those of the genome overall.

In addition, unlike the experiments of Terleth *et al.*, these repair rates were determined during liquid holding conditions to eliminate DNA synthesis. We note that in our experiments we did not eliminate DNA that was synthesized during the incubations after UV irradiation and that this could lead to an overestimate of removal of CPDs. However, there should not be a significant amount of replication during our experiments given the short incubation periods (≈1/4 of a generation time), growth rates of the strains used, and the UV doses given. In addition, the overestimate of repair due to replication should affect both strands.

Our results show that preferential repair of the transcribed strand of *RPB2* in the chromosome is similar to that in a plasmid and more closely resembles the strand bias for repair observed for the multicopy plasmid TRURAP (10) than the repair observed at the mating-type loci (7). We note that the repair rates reported by Terleth *et al.* (7) are much slower than those observed by others (10, 11, 22). The slower repair rates observed might be strain-specific or due to low levels of transcription from *MATα*.

A current model for the transcription-associated DNA repair of UV photoproducts is that the transcription machinery stalls at a lesion and serves as a signal to direct the repair enzymes to the lesion site (5). This could imply a mechanism common to *E. coli* and eukaryotes in which repair enzymes recognize a blocked RNA polymerase, perhaps with the aid of a coupling factor (6, 26). Due to the similarity among the three RNA polymerases in eukaryotes (27), a stalled RNA pol I or pol III complex might also be expected to serve as an antenna for repair enzymes, as RNA pol II appears to do. The lack of preferential repair of genes transcribed by RNA pol I (Fred Christians and P.C.H., unpublished data) and perhaps RNA pol III (28) in eukaryotes implies that there could be a factor that specifically interacts with RNA pol II. Alternatively, sequestering repair enzymes to particular regions within the nucleus where RNA pol II is transcribing (29) could also explain the particular dependence on this polymerase.

Elucidation of the mechanism of transcription-associated DNA repair will require the purification and characterization of the proteins involved. Purification of a protein that appears to couple repair with transcription has recently been achieved in *E. coli* (26) and this protein is defective in mutation frequency decline mutants (*mfd⁻*) (30). The biochemical characterization of this factor that could couple repair to the transcription machinery may provide insights into DNA excision repair in *E. coli*.

We suggest another possible mechanism that might be operating in eukaryotes for recognition of damage in the transcribed strands of active genes, in which DNA repair enzymes, having a high affinity for single-stranded nucleic acids, could bind the nascent RNA and, with their intrinsic helicase activities (31–33), proceed into and unwind the DNA·RNA hybrid, thereby displacing the stalled RNA polymerase. The helicase might then remain associated at the lesion site in the template strand. Additional proteins of the repair complex may then assemble upon the helicase to initiate removal of the lesion. In such a model, the recognition protein for the blocked transcription complex would be expected to be bifunctional—to be able to bind RNA and displace RNA from a DNA·RNA hybrid. A protein with the appropriate characteristics has recently been reported. The yeast gene *SSL2* (Suppressor of Stem–Loop Mutation), encoding a protein that binds RNA, has been shown to be highly homologous to the human *ERCC-3* gene, the protein product of which is defective in a patient with xeroderma pigmentosum group B and Cockayne syndrome (34). In Cockayne syndrome the repair deficiency is in the preferential repair of expressed genes (ref. 35; Isabel Mellon and P.C.H., unpublished data). The DNA sequence of *SSL2* contains motifs

common to helicases and, therefore, may have helicase activity. Biochemical characterization of proteins like *SSL2*, which might direct repair enzymes to lesion sites in expressed genes, may help elucidate the mechanisms by which excision repair is associated with transcription in eukaryotes.

Note Added in Proof. We have now compared rates of removal of CPDs from *RPB2* in the wild-type parent strain YSH10 in the presence and absence of cycloheximide (100 μg/ml). No effect on repair was detected. Thus, we have eliminated the possibility that the lack of preferential repair of the transcribed strand of *RPB2* in the temperature-sensitive strain YSH11 at the nonpermissive temperature could be due to loss of function of a labile protein. Furthermore, this indicates that the existing level of repair enzymes in the absence of further protein synthesis is sufficient for preferential repair of the transcribed strand in expressed genes—i.e., induction is not necessary.

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