Transcription-coupled repair in yeast is independent from ubiquitylation of RNA pol II: Implications for Cockayne's syndrome

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Cockayne's syndrome cells lack transcription-coupled nucleotide excision repair (TCR) and ubiquitylation of RNA polymerase II large subunit (RNA pol II LS), suggesting that ubiquitylation of RNA pol II LS may be necessary for TCR in eukaryotes. Rsp5 is the sole yeast ubiquitin-protein ligase that ubiquitylates RNA pol II LS in cells exposed to DNA-damaging agents. In yeast lacking functional Rsp5, there is no ubiquitylation of RNA pol II LS. We show here that removal, repression, or over-expression of Rsp5 has no effect on TCR, demonstrating that ubiquitylation of the RNA pol II LS is not required for TCR. We infer that the lack of ubiquitylation of RNA pol II LS in Cockayne's syndrome cells does not cause their defect in TCR.

Cockayne's syndrome (CS) is a rare autosomal recessive disorder displaying a variety of symptoms, including severe neurological abnormalities, dwarfism, deficiency of subcutaneous fat, and sun sensitivity (1). The UV sensitivity of CS patients and their cells in culture is thought to result from a deficiency in RNA synthesis (2), likely because of an inability to preferentially remove transcription-blocking DNA damage from the transcribed strands of expressed genes: i.e., impaired transcription-coupled repair (TCR) (3, 4).

It has been shown that HeLa cells and normal human fibroblasts in culture ubiquitylate the large subunit of RNA polymerase II (RNA pol II LS) after exposure to UV radiation, cisplatin, mitomycin C, and methyl methanesulfonate (5). The ubiquitylation of RNA pol II LS was absent in UV-irradiated fibroblasts from CS patients. Interestingly, fibroblasts from patients displaying another autosomal recessive disorder associated with a DNA repair deficiency, xeroderma pigmentosum, were capable of ubiquitylating RNA pol II LS after UV irradiation (6). In both normal and xeroderma pigmentosum fibroblasts, the ubiquitylated form of RNA pol II LS was hyperphosphorylated, a form that is associated with the elongating transcription complex.

Ubiquitin is a 76-amino acid peptide that gets linked via its terminal glycine (residue 76) to a cysteine residue of the ubiquitin-activating protein (E1) via a thioester bond. The ubiquityl moiety is then moved via transesterification from the E1 enzyme to a cysteine residue of one of the ubiquitin-conjugating enzymes (E2). Ubiquitin is then transferred via further transesterification to one or more ε-lysine residues in the acceptor or target protein. This step may require a ubiquitin-protein ligase, or E3. Finally, the recently identified E4 proteins bind ubiquitylated proteins and, together with E1, E2, and E3, facilitate formation of multiubiquitin chains on substrate proteins (7, 8). Substrates of the ubiquitin system are degraded by the large 26*S* proteasome in an ATP-dependent fashion. Ubiquitylation may also serve a regulatory function independent of proteolysis. Ubiquitylation, through proteolysis and other mechanisms, plays a regulatory role in the cell cycle, cellular differentiation, stress responses, and many other cellular processes.

In the yeast *Saccharomyces cerevisiae*, the *RSP5* gene encodes an essential E3 enzyme that is a member of the HECT domain family of ubiquitin-protein ligases (9). One of the functions of Rsp5 is to ubiquitylate RNA pol II LS after exposure of the cells to DNA-damaging agents (10). Exposing yeast to UV radiation or UV-mimetic 4-NQO, two genotoxic agents that generate DNA adducts that are removed by nucleotide excision repair (NER), results in ubiquitylation and degradation of RNA pol II LS (11). Recently, Huibregtse and colleagues demonstrated that, in yeast strains lacking Rsp5 function, there is no ubiquitylation of RNA pol II LS after exposure to 4-NQO (11). The finding that RNA pol II LS is ubiquitylated in yeast and humans suggests a possible regulatory mechanism for cellular processes that require rapid turnover of RNA pol II. For example, repair of the transcribed DNA strand might be facilitated by rapid clearance of blocked transcription complexes. This clearance might be accomplished by ubiquitylation and subsequent degradation of RNA pol II LS. Bregman and colleagues suggested a plausible model describing the role for ubiquitylation in TCR (5, 6). It was proposed that the transcription complex synthesizes mRNA until it encounters a DNA adduct in the transcribed strand of an expressed gene. The transcription complex arrests at the adduct until a ubiquitin-protein ligase binds RNA pol II LS via the CTD and ubiquitylates RNA pol II LS. The ubiquitylated RNA pol II LS is then displaced from the DNA template and degraded by the 26*S* proteasome. To test this model, we determined the influence of ubiquitylation of RNA pol II LS on TCR and genomic NER in yeast.

Materials and Methods

Media, Plasmids, and Strains. All media were prepared as described by Adams *et al.* (12). Yeast extract/peptone/dextrose medium is 1% yeast extract/2% Bacto-peptone (Difco)/2% glucose. Yeast extract/peptone/galactose medium is 1% yeast extract/2% Bacto-peptone (Difco)/2% galactose/1% raffinose. Synthetic glucose medium (SD) is 2% glucose/0.67% Bacto-yeast nitrogen base without vitamins (Difco) supplemented with the appropriate amino acids and bases. Agar (1.5%) was added to media for plates. Yeast strains used in this study were FY56 (*MAT*α *his4-912*δ*R5 lys2-128*δ *ura3-52*), FY1808 (*MAT*α *his4-912*δ*R5 lys2-128*δ *ura3-52 rsp5-1*), GAL-RSP5 (*MAT*α *his4-912*δ*R5 lys2-128*δ *URA3 GALRSP5*) (9) (kindly provided by Jon Huibregtse, Rutgers University), and MGSC102 (a strain containing *RAD26* disrupted with *HIS3* in the W303-1B genetic background) (13) (kindly provided by A. van Gool, Organon Nederland bv). Plasmid pKS212 is a pBluescript KS (+) vector (Stratagene) into which the internal 1.0-kb *Eco*RI-*Xho*I fragment from *RPB2* was

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Abbreviations: TCR, transcription-coupled nucleotide excision repair; RNA pol II LS, RNA polymerase II large subunit; NER, nucleotide excision repair; CS, Cockayne's syndrome; CPD, cyclobutane pyrimidine dimer.

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inserted (14). Plasmid pKS212 was linearized by cleaving with *Xho*I or *Eco*RI and was incubated with rNTPs, [^{32}P]αCTP (Amersham Pharmacia), and T7 RNA polymerase or T3 RNA polymerase, respectively, under conditions recommended by the manufacturer to generate strand-specific RNA probes for *RPB2*.

Growth and UV Irradiation of Yeast Cells. Growth conditions were as described by Huibregtse and colleagues (9–11), and irradiation of strains was as described previously (15). Photoreactivation was avoided by performing all manipulations under yellow light. Exponentially growing cultures at 30°C or 37°C were collected by centrifugation and were resuspended in ice-cold PBS at 1×10^7 cells/ml. Shaking cell suspensions ($\approx$0.2 cm deep to ensure a uniform UV dose to all cells) were irradiated with predominantly 254-nm UV light at 0.33 J/m^2/s by using an American Ultraviolet (Murray Hill, NJ) germicidal lamp. The cells were collected by centrifugation after irradiation and were either lysed immediately or resuspended in their original growth media at 30 or 37°C. Cells were incubated for various times to allow DNA repair and then were lysed.

Isolation of Yeast DNA. After digestion with Zymolyase 100T, spheroplasts were collected by centrifugation and were resuspended in 0.2 ml of Zymolyase buffer lacking Zymolyase (15). Spheroplasts were then diluted with 2.8 ml of 0.05 M Tris·HCl (pH 8.5)/0.05 M EDTA and were lysed by the addition of 0.2 ml of 20% sarkosyl (16). The mixture was then chilled on ice for >10 min. Cellular debris and sarkosyl were precipitated by the addition of 0.64 ml of 5 M potassium acetate. Mixtures were incubated at 4°C overnight and were centrifuged at 4,500 rpm in a Sorvall H6000A rotor at 4°C for 25 min. Supernatants containing chromosomal DNA were transferred to fresh tubes and were precipitated by addition of two volumes of ice-cold ethanol, and pellets were washed with ice-cold 70% ethanol (17). Samples were then resuspended in 10 mM Tris·HCl (pH7.5)/1 mM EDTA (TE) and were treated with RNaseA (final concentration, 50 μg/ml), and the DNA was digested to completion with *Pvu*I and *Pvu*II restriction enzymes. The purified, restricted DNA was ethanol precipitated, was resuspended in TE, and was stored at 4°C.

Strand-Specific Analysis of Frequency of Cyclobutane Pyrimidine Dimers (CPDs). The incidence of CPDs in a particular restriction fragment was determined by methods previously developed (18, 19). In brief, purified and restricted DNA [0.2 μg in 10 mM Tris·HCl (pH 7.5)/0.1 M NaCl/10 mM EDTA/1 mg/ml BSA) was mock-treated or digested with T4 endonuclease V, a CPD-specific DNA glycosylase/AP lyase, in 40 μl for 30 min at 37°C. Digestion of DNA samples by T4 endonuclease V was stopped by the addition of 10 μl of 12.5% Ficoll/5 mM EDTA/0.125% bromophenol blue/0.25 M NaOH. Samples were loaded into 0.5% alkaline agarose gels and were electrophoresed at 1.7 V/cm overnight with recirculating buffer (30 mM NaOH/1 mM EDTA). DNA was transferred to Hybond N$^+$ (Amersham Pharmacia). Membranes were prehybridized for at least 2 h, then were hybridized with strand-specific RNA probes made from pKS212. Autoradiographic signal intensities were quantified and analyzed by using a Hewlett–Packard Scanjet IIcx, DESKSCAN II, and NIH IMAGE 1.62.

Results and Discussion

We examined repair in a well-characterized *GAL-RSP5* yeast strain (GAL-RSP5) expressing or not expressing the ubiquitin-protein ligase Rsp5, the enzyme that ubiquitylates the large subunit of RNA pol II after exposure to DNA-damaging agents (9, 11). Growth of strain GAL-RSP5 in galactose-containing medium results in greater than 30-fold induction of Rsp5 (9, 10). In contrast, incubation of this same strain in glucose-containing

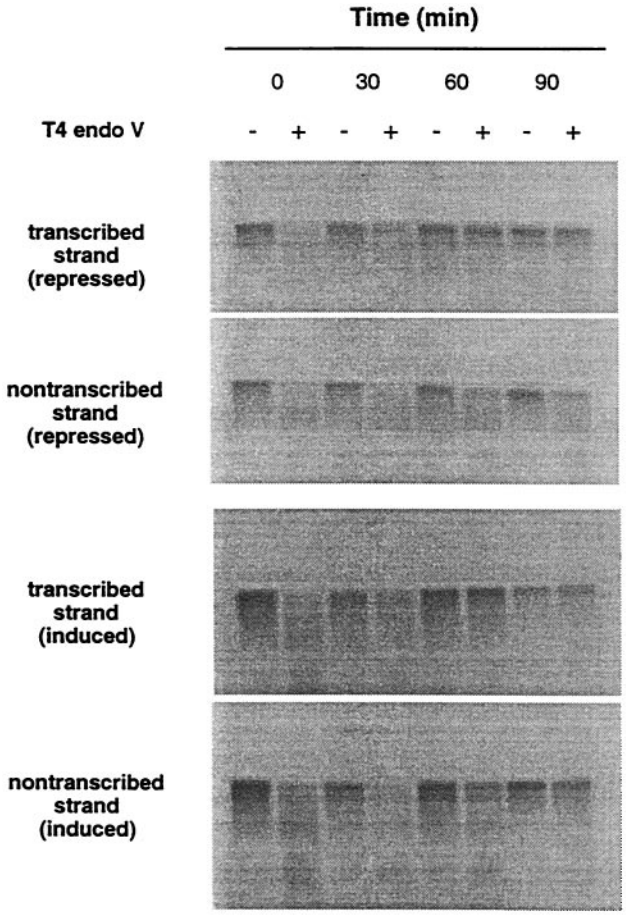

Fig. 1. Autoradiograms demonstrating repair in each strand of the *RPB2* gene in a strain containing a galactose-inducible *RSP5* gene, *GAL-RSP5*. Exponentially growing cultures at 30°C were induced with galactose or were repressed with glucose for 18–22 h, were UV-irradiated and incubated in growth medium at 30°C for the times indicated. UV irradiations were with 60 J/m^2. DNA purified from the cells was digested with *Pvu*I and *Pvu*II restriction endonucleases. A portion (0.2 μg) of restricted DNA was digested with T4 endonuclease V or was mock-treated, then was electrophoresed through 0.5% alkaline agarose. DNA was transferred to Hybond N$^+$ membrane and was hybridized with an RNA probe specific for the nontranscribed strand of the *RPB2* gene, and an autoradiogram was generated. The probe was removed, and the immobilized DNA was then hybridized with an RNA probe specific for the transcribed strand. The autoradiograms show the 5.3-kb *Pvu*I-*Pvu*II restriction fragment.

medium results in repression of Rsp5 to undetectable levels (9, 10). Lack of Rsp5 results in loss of ubiquitin-mediated degradation of RNA pol II LS, which is evidenced by a 4- to 5-fold accumulation of RNA pol II LS (9, 10). Exponentially growing cultures in inducing (galactose) medium were harvested by centrifugation, washed, and resuspended in repressing (glucose) medium or inducing (galactose) medium. Cultures were incubated under these conditions for 18–22 h and 48 h, then were irradiated as described in ref. 15 and in *Materials and Methods*. Repair of the transcribed strand is robust under both inducing or repressing conditions (Fig. 1). Sixty minutes after UV irradiation, approximately 90% of the CPDs had been removed from the transcribed strand of *RPB2*. During this same period, approximately 50% of the CPDs had been removed from the nontranscribed strand of *RPB2* (Fig. 2*A*). Thus, repair of the transcribed strand was faster than repair of the nontranscribed strand; i.e., TCR was present in cells overexpressing or lacking Rsp5. We note that this repair is similar to the repair we

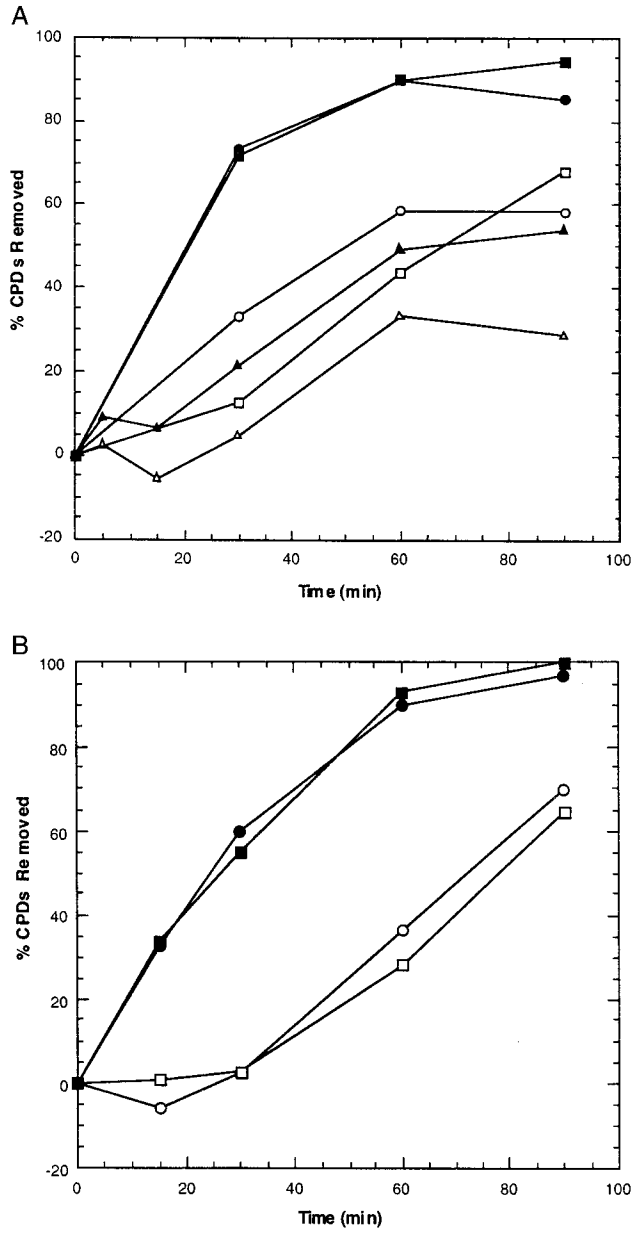

Fig. 2. (*A*) Time course for removal of CPDs from each of the strands of the *RPB2* gene in a strain containing a galactose-inducible *RSP5* gene, *GAL-RSP5*, and a strain defective for TCR, MGSC102 (*rad26*::*HIS3*). Exponentially growing cultures of GAL-RSP5 at 30°C were induced with galactose or repressed with glucose for 18–22 h, UV-irradiated with 60 J/m^2, and incubated in galactose or glucose growth medium at 30°C for the times indicated. Exponentially growing cultures of the *rad26* mutant (MGSC102) were grown in glucose-containing medium and were irradiated with 30 or 60 J/m^2. Repair was determined from the measured incidences of CPDs in each strand of the *Pvu*I-*Pvu*II restriction fragment of the *RPB2* gene. Data points for GAL-RSP5 represent the average of three independent experiments. Data points for MGSC102 represent the average of four independent experiments. GAL-RSP5, transcribed strand, galactose (■); GAL-RSP5, nontranscribed strand, galactose (□); GAL-RSP5, transcribed strand, glucose (●); GAL-RSP5, nontranscribed strand, glucose (○); MGSC102, transcribed strand (▲; MGSC102, nontranscribed strand (△). (*B*) Time course for removal of CPDs from each of the strands of the *RPB2* gene in the parent strain FY56. Exponentially growing cultures at 30°C were incubated with galactose or glucose for 22 h, UV-irradiated with 60 J/m^2, and incubated in galactose or glucose growth medium at 30°C for the times indicated. Repair was determined from the measured incidences of CPDs in each strand of the *Pvu*I-*Pvu*II restriction fragment of the *RPB2* gene. Transcribed strand, galactose (■); nontranscribed strand, galactose (□); transcribed strand, glucose (●); nontranscribed strand, glucose (○).

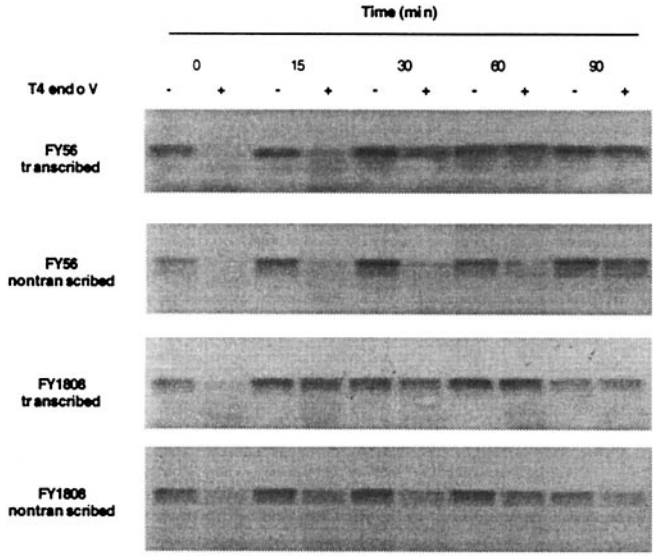

Fig. 3. Autoradiograms demonstrating proficient repair from each of the strands of the *RPB2* in a heat-sensitive *rsp5-1* (FY1808) mutant and the wild-type parental strain (FY56). Exponentially growing cultures at 30°C were shifted to 37°C for 2 h, and then were UV-irradiated with 60 J/m^2 and incubated in growth medium at 37°C for the times indicated. Cells were lysed and DNA was purified for repair analysis as described in *Materials and Methods*.

observed in the parental strain (FY56) under the same conditions (Fig. 2*B*). Similar repair rates were observed for cultures incubated in inducing or repressing conditions for 48 h (data not shown).

Repair in a strain defective for TCR (MGSC102) is shown for comparison. The strain contains *RAD26* disrupted with *HIS3* in the W303-1B genetic background (13). In the *rad26* mutant, repair of the transcribed strand of *RPB2* was reduced almost to the level of repair of the nontranscribed strand (Fig. 2*A*). We find that *RPB2* is a reliable reporter gene in which to measure TCR and exhibits similar repair rates as other genes transcribed by RNA pol II at basal levels (data not shown).

The GAL-RSP5 strain used above also contains an allele of *RSP5* lacking the catalytic HECT domain. The expression of the truncated *RSP5* allele is under the control of the endogenous *RSP5* promoter and is likely to be increased after exposure to DNA damaging agents (20). The truncated Rsp5 protein is still capable of binding physiological substrates via the WW domains (9, 10). In addition, long periods of incubation under repressing conditions are needed to reduce Rsp5 to undetectable levels, which would be expected to affect other cellular functions that might indirectly impinge on TCR and/or NER. To avoid complications associated with expression of the truncated allele of *RSP5* and the long term repression of *GAL-RSP5*, we next examined TCR and NER in a yeast strain, FY1808, containing a temperature-sensitive allele of *RSP5*, *rsp5-1*. This temperature-sensitive allele was first isolated by F. Winston and colleagues and has been clearly shown to rapidly lose Rsp5 ubiquitin-protein ligase activity when shifted to the nonpermissive temperature (10, 11). In the temperature-sensitive *rsp5-1* strain, after exposure to a DNA-damaging agent, there is no ubiquitylation of RNA pol II LS by Rsp5 or any alternate pathway after 1–2 h at the nonpermissive temperature (10, 11).

Exponentially growing cultures of FY1808(*rsp5-1*) and its isogenic parent FY56(*RSP5*) at 30°C were shifted to the nonpermissive temperature (37°C) for 1 or 2 h before exposure to UV radiation, the same experimental conditions in which ubiquitylation of RNA pol II LS is clearly absent (11). The autoradiogram from a repair experiment with FY56 and FY1808 at

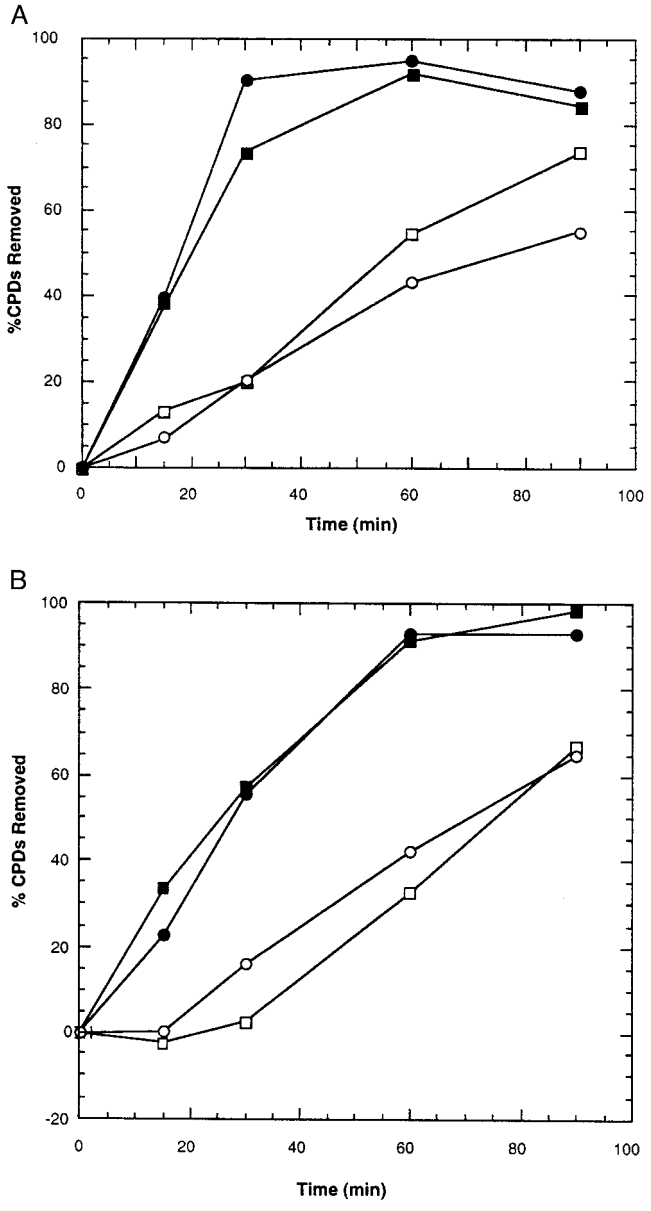

Fig. 4. (*A*) Time course for removal of CPDs from each of the two strands of *RPB2* in a heat-sensitive *rsp5-1* (FY1808) mutant and the wild-type parental strain (FY56). Exponentially growing cultures at 30°C were shifted to 37°C for 2 h, and then were UV-irradiated with 60 J/m^2 and incubated in growth medium at 37°C for the times indicated. (*B*) Exponentially growing cultures at 30°C were UV-irradiated with 60 J/m^2 and incubated in growth medium at 30°C for the times indicated. Repair was determined from the measured incidences of CPDs in each strand of the *Pvu*I-*Pvu*II restriction fragment of the *RPB2* gene. Data points represent the average of three independent experiments. Transcribed strand, FY56 (■); nontranscribed strand, FY56 (□); transcribed strand, FY1808 (●); nontranscribed strand, FY1808 (○).

37°C is shown in Fig. 3. Hybridization signals were quantified by densitometric scanning and are presented in Fig. 4*A*. Repair of the transcribed strand is rapid for both FY56 and FY1808; i.e., all repair is complete by 60 min after UV irradiation. Repair of the nontranscribed strand of *RPB2* is the same in both FY56 and FY1808, and it is slower than the repair in the transcribed strand. Sixty minutes after UV irradiation, approximately 50% of the CPDs were removed from the nontranscribed strand. The repair we observed at 37°C is similar to the repair we observed at 30°C, the permissive temperature for *rsp5-1* (Fig. 4*B*). We note that repair of the nontranscribed strand of a gene is a reliable indicator of overall genomic repair: i.e., NER. Thus, the heat-sensitive *rsp5-1* mutant has no defect in TCR or NER.

It is still possible that some other E2 or E3 enzyme is carrying out ubiquitylation of RNA pol II LS in the Rsp5-deficient strains. However, there does not appear to be an alternative pathway for ubiquitylation of RNA pol II LS. In the *rsp5-1* mutant at the nonpermissive temperature, there is no ubiquitylation of RNA pol II LS after exposure to 4-NQO (11). If there were an alternative pathway for ubiquitylation and degradation of RNA pol II LS, then ubiquitylated RNA pol II LS should be present and degradation of RNA pol II LS should occur in the *rsp5-1* mutant at the nonpermissive temperature. Contrary to this prediction, there is no apparent proteolysis of RNA pol II LS in *rsp5-1* at the nonpermissive temperature. Thus, under the conditions used in this report, there is no alternative pathway for proteolysis of RNA pol II LS.

Taken together, our data demonstrate that ubiquitylation of RNA pol II LS is not required for TCR. How is it that cells from CS patients are defective in TCR and in ubiquitylation of RNA pol II LS and yet ubiquitylation of RNA pol II LS is not required for TCR? It appears that ubiquitylation and degradation of RNA pol II LS occurs independently of, or subsequently to, repair of DNA damage in the transcribed strands of genes. By analogy, the defect in TCR observed for cells from CS patients is probably not attributable to their lack of ubiquitylation of RNA pol II LS in response to DNA damage. We propose a model in which, in cells possessing DNA damage, transcription elongation occurs until a DNA lesion is encountered. At this point, the elongation complex stalls, and in normal cells the stalled complex is removed. This frees the RNA pol II LS from the chromatin, and it is this free form that is the substrate for ubiquitylation and degradation. It is likely that cells from CS patients have a defect in their ability to remove proteins bound to chromatin; i.e., RNA pol II LS is not removed from DNA and, therefore, would not be ubiquitylated.

Support for our model comes from at least two lines of evidence. First, ubiquitylation of RNA pol II LS by Rsp5 has not been shown to take place in the nucleus. There is some evidence that it occurs instead at the nuclear envelope, endoplasmic reticulum, or in the cytoplasm (21, 22). A cytoplasmic locale for Rsp5 activity is consistent with observations for two membrane-associated proteins that are ubiquitylated by Rsp5 in yeast. Permeases Gap1p and Fur4p are ubiquitylated at the cell membrane before being internalized by endocytosis (23–25). In addition, the human and mouse homologs of Rsp5, Nedd4, have been shown to be exclusively cytoplasmic (26, 27). It will be interesting to determine the subcellular distribution of Rsp5. If it is cytoplasmic, it would further support the notion that the blocked transcription elongation complex must be removed from the DNA before RNA pol II LS can be ubiquitylated by Rsp5 and degraded. The second line of evidence supporting our model suggests that cells from CS patients may have a defect in their ability to remove proteins bound to chromatin because the CSB protein and its yeast homolog, Rad26, have homology to members of the Swi2/Snf2 family of DNA-dependent ATPases (28). Several members of this family are proteins with demonstrated roles in chromatin remodeling and/or removing protein bound to chromatin (29–32). We are currently determining whether Rad26 is indeed involved in chromatin remodeling.

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