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Transcription Preferentially Inhibits Nucleotide Excision Repair of the Template DNA Strand *in Vitro**

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It has been reported that pyrimidine dimers (pyrimidine<>pyrimidine) are removed preferentially from actively transcribing genes. Furthermore, the preferential repair is restricted to the transcribed strand of these genes. Currently there is no mechanistic explanation for these phenomena. In this study we investigated the effect of transcription on nucleotide excision repair using defined *Escherichia coli* systems consisting of DNA substrates containing a strong promoter and either (*a*) a T<>T at a defined position in the nontranscribed or transcribed strand or (*b*) photoproducts randomly distributed in both strands, as well as transcription and nucleotide excision repair enzymes. While a T<>T in the nontranscribed strand had no effect on transcription, a photodimer in the transcribed strand blocked transcription causing RNA polymerase to stall at the T<>T site. This stalled elongation complex inhibited the excision of the photodimer by (A)BC excinuclease resulting in a net effect of preferential repair of the nontranscribed strand in a mixture containing both substrates. Similarly, when we conducted transcription/repair experiments with a superhelical plasmid no enhanced repair of the transcribed gene was observed compared to nontranscribed regions. We conclude that RNA polymerase stalled at a photodimer does not direct the (A)BC excinuclease to the damaged template strand and therefore cannot account for the strand-specific repair observed *in vivo*.

Bohr *et al.* (1985) discovered that pyrimidine dimers in the dihydrofolate reductase gene of Chinese hamster ovary cells were removed four to five times more rapidly compared to nontranscribed regions of the chromosome. Mellon *et al.* (1987) demonstrated that this enhanced repair was due almost exclusively to the preferential repair of the transcribed strand. More recently Terleth *et al.* (1989) and Mellon and Hanawalt (1989) showed preferential repair in transcribed genes of *S. cerevisiae* and *Escherichia coli*, respectively. The latter authors measured the rate of repair of pyrimidine dimers in the two strands of the *lac* operon following UV (254 nm) irradiation. They found that when the operon was actively transcribed, the photodimers were removed five to 10 times more rapidly from the transcribed strand compared to the nontranscribed strand; no difference was found in the rate of repair of the two strands in uninduced cells.

Initially, preferential repair of transcribed genes was ascribed to a more open conformation of the transcribed regions of chromatin (see Bohr *et al.*, 1988). However, the discovery that preferential repair is limited to the transcribed strand suggests a more specific coupling mechanism between transcription and repair. A simple model for this coupling proposed by Mellon and Hanawalt (1989) is as follows. RNA polymerase transcribes a gene until it reaches a UV photoproduct; transcription then halts, the RNA polymerase makes a stable elongation complex at the transcriptional stopsite, and this complex constitutes a high affinity site for the nucleotide excision repair proteins which remove the photodimer. A precedent for the targeting of UV photoproducts in DNA for repair by a lesion-bound protein exists. Photolyase, which binds to the pyrimidine dimer-containing strand of the duplex (Husain *et al.*, 1987), stimulates the excision of the photodimer by (A)BC excinuclease, the enzyme that initiates nucleotide excision repair in *E. coli* (Sancar *et al.*, 1984; Sancar and Sancar, 1988; Orren and Sancar, 1989).

The above model is an oversimplification of what takes place in an *E. coli* cell as it neglects the topological effects of transcription (Gamper and Hearst, 1982; Tsao *et al.*, 1989), transcription factors, the effect of transcription-coupled translation and other phenomena as possible contributors in directing repair enzymes to the transcribed strand. Nevertheless, the simple model outlined above is testable *in vitro*. In this study we have used either a 137-mer duplex containing a single thymine photodimer located on the nontranscribed or the transcribed strand downstream from a *tac* promoter, or a UV-irradiated superhelical plasmid containing a *tac* promoter in a coupled transcription/repair system which includes *E. coli* RNAP,[1] (A)BC excinuclease, and DNA polymerase I and ligase when necessary to study the effect of transcription upon repair. We find that under these *in vitro* conditions, the template strand is repaired less efficiently than the nontranscribed strand.

MATERIALS AND METHODS

Enzymes and Substrates

E. coli RNA polymerase (σ factor content not less than 50%) and the RNase inhibitor RNasin were purchased from Promega Biotec (Madison, WI), DNA polymerase I, T4 DNA ligase, restriction enzymes, and DNase I were from Bethesda Research Laboratories, and ribonuclease *Bacillus cerus* and ribonuclease T1 were from Pharmacia LKB Biotechnology Inc. The *E. coli* photolyase was purified as described by Sancar *et al.* (1984) and the UvrA, -B, and -C proteins

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05700.

[1] The abbreviations used are: RNAP, RNA polymerase; TRB, transcription-repair buffer; T<>T or thymine photodimers, thymine-thymine cyclobutane photodimer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); kb, kilobase(s).

were purified by the method of Thomas *et al.* (1985).

Two types of substrates were used. One was a 137-mer duplex which contained a *tac* promoter and a thymine photodimer (T<>T) in either the transcribed or the nontranscribed strand. The 137-mers labeled with ^{32}P at the 5′ end of the strand with T<>T were constructed by ligating the eight individual oligomers shown in Fig. 1 as described previously for a psoralen-adducted substrate (Shi *et al.*, 1987 and 1988; Van Houten *et al.*, 1987). The central 11-base oligomer with the T<>T was prepared and purified by the method of Banerjee *et al.* (1988). The second type of substrate for transcription-repair experiments was superhelical pDR3274 plasmid irradiated with 75 Jm^{-2} of 254-nm UV light from a Quantacount monochrometer (Photon Technology, Inc., Princeton, NJ). This UV dose produces about 2 UV photoproducts (cyclobutane dimers and 6-4 photoproducts) per kilobase pair (estimated by incision of cyclobutane dimers and assuming 6-4 photoproducts are 10% of total photoproducts). pDR3274 is a pBR328 derivative which contains, in addition to a functional *tet* gene, the *uvrC* gene under the control of a *tac* promoter (Thomas *et al.*, 1985).

Experiments with the Linear Substrate

Transcription—The transcription buffer contained 40 mM Tris-HCl, pH 7.95, 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, and RNasin at 0.8 unit/μl. Reaction mixtures (12.5 μl) containing about 0.5–5.0 fmol of end-labeled DNA, RNAP (RNA polymerase), and 1.3 μM [α-^{32}P]UTP (3 Ci/μmol, Du Pont-New England Nuclear) were incubated at 37 °C for 10 min to form initiation complexes. Then 12.5 μl of transcription buffer (at 37 °C) containing GTP, CTP, and UTP (unlabeled) at 400 μM each was added and the reaction mixtures were incubated at 37 °C for an additional 8 min. The reactions were stopped by adding EDTA to 10 mM, sodium dodecyl sulfate to 0.2%, $LiCl_2$ to 100 mM, and 0.1 mg of oyster glycogen. The nucleic acids were precipitated with ethanol, resuspended in a formamide dye solution, and analyzed on 8% polyacrylamide sequencing gels.

DNase I Footprinting of RNA Polymerase—Reaction mixtures (25 μl) contained about 0.5 fmol of DNA and 0–66 nM RNAP in transcription buffer with or without 200 μM each CTP, GTP, and UTP. The reaction mixtures were incubated at 37 °C for 10 min, $CaCl_2$ was then added to 5 mM and the DNA was digested with 20 pg of DNase I for 6 min. Cold oyster glycogen (100 μg in 25 μl) was then added as carrier and the DNA was precipitated with ethanol and analyzed on 8% polyacrylamide sequencing gels.

Incision by (A)BC Excinuclease—The incision reactions were conducted in 100-μl volumes with about 1 fmol of DNA, 4.2 nM UvrA, 63 nM UvrB, and 140 nM UvrC; CTP, GTP, and UTP each at 200 μM; and, when indicated, 1.2–230 nM *E. coli* photolyase or 0.3–66 nM RNAP. RNAP or photolyase were incubated with the DNA for 5–10 min at 37 °C before addition of (A)BC excinuclease unless otherwise indicated. The mixtures were further incubated at 37 °C for 8 or 15 min unless otherwise indicated and then the reactions were stopped and the products were analyzed as under "DNase I Footprinting of RNA Polymerase."

RNA Sequencing—Purified truncated transcripts were subjected to sequence-specific degradation using ribonuclease *B. cerus* and ribonuclease T1 and instructions provided by the supplier. RNA ladders were generated from purified full-length transcripts by heating at 90 °C in 50 mM bicarbonate buffer (pH 9.0) for 15 min to 2 h (Donis-Keller *et al.*, 1977). Reaction products in urea dye loading mixtures were analyzed on 11 and 20% polyacrylamide sequencing gels.

Experiments with Superhelical Substrate

Quantitative Analysis of Transcription—Transcription was carried out in transcription-repair buffer (TRB) which contained 40 mM HEPES, pH 7.8, 50 mM KCl, 7.4 mM $MgCl_2$, 2 mM ATP, 3.4% glycerol, 0.9 mM dithiothreitol, and 0.8 unit/μl RNasin and, when indicated, dNTPs at 40 μM each (except dCTP at 10 μM) and rNTPs at 200 μM each. For quantitative analysis of transcription, initiation complexes were formed by incubating RNAP with 70 fmol of plasmid and 1.3 μM of unlabeled UTP in 25 μl of TRB for 5 min at 37 °C. Then, 25 μl of TRB at 37 °C containing 400 μM each of CTP, GTP, and UTP plus 66 nM [α-^{32}P]UTP was added. The reaction mixtures were incubated at 37 °C and the reactions were stopped by adding 50 μl of cold 10% trichloroacetic acid. The volume was adjusted to 1 ml with 5% trichloroacetic acid, and RNA was collected by centrifugation and two washes with 5% trichloroacetic acid. The RNA was then dissolved with 1 ml of 1 M Tris-HCl, pH 8.0, containing trace amounts of RNase A and quantified by scintillation counting.

Repair Synthesis—Repair synthesis reactions were conducted in TRB containing 0.4 unit/μl RNasin. Initiation complexes were formed by incubating DNA (about 140 fmol) with RNA polymerase (13 pmol) and UTP (5 μM) in 50 μl at 37 °C for 5 min. Transcription and repair were started by adding 50 μl of a prewarmed solution in TRB to bring final concentrations of CTP, GTP, and UTP to 200 μM each, dATP, dGTP, and TTP to 40 μM each, dCTP to 10 μM including 2–8 μCi of [α-^{32}P]dCTP, UvrA to 1.4 nM, UvrB to 16 nM, UvrC to 30 nM, *PolI* to 200 nM, and T4 DNA ligase to 20 units/ml. Under these conditions we found the UvrA and photoproduct concentrations to be limiting factors, *i.e.* there was an increase in repair synthesis with higher UvrA concentrations or UV doses (data not shown). Reactions were stopped after 8 min at 37 °C by mixing each with a 100 μl volume of phenol. The DNA was extracted with phenol and ether and precipitated with ethanol. Following digestion with *Eco*RI, the DNA was further purified on 0.8% agarose gels, electroeluted from gel slices, precipitated with ethanol, resuspended, dialyzed, digested with *Bgl*II and *Hin*dIII and the resulting fragments were separated on 1.2% agarose gels. The gels were stained with ethidium bromide, and the DNA was visualized and photographed on a UV light box. Gels were then dried and autoradiographed. Following autoradiography, the gels were rehydrated, the DNA bands were excised, and the agarose was digested with 1 ml of 1 N perchloric acid at 65 °C for 1–3 h, 0.1 ml of 10 N NaOH was added to neutralize the samples, and the radioactivity was measured by scintillation counting. The relative amounts of DNA recovered were determined by scanning (using a Zeineh Softlaser Scanning Densitometer model SLR-2D/1D) photographs of ethidium bromide-stained gels and the amounts of radiolabel incorporation into DNA were normalized for the amount of DNA recovered in each lane.

RESULTS

We used two types of substrate to investigate the effect of transcription on nucleotide excision repair. One of the substrates was a 137-mer duplex containing a strong *tac* promoter and a single thymine photodimer in either the nontranscribed or the transcribed strand (Fig. 1). Using this substrate we were able to examine effects of template and nontemplate lesions on transcription and consequences for repair. The second substrate was a UV-irradiated superhelical plasmid which contained the *tac* promoter from which a gene (*urvC*) constituting approximately one third of the plasmid is transcribed (Thomas *et al.*, 1985). This substrate was used to examine, by the repair synthesis assay, whether the transcribed sequences were repaired more efficiently than nontranscribed sequences and whether transcription had any effect on the rate and extent of repair in the transcribed and nontranscribed regions of the plasmid.

Transcription and Repair of a Linear Substrate Containing a Thymine Dimer at a Defined Site

Transcription—It has been known for a long time that pyrimidine dimers block transcription in both pro- and eucaryotic cells (Sauerbier and Hercules, 1978). We constructed a synthetic DNA fragment ("T" in Fig. 1) with a strong promoter (*tac*) and a thymine dimer in the transcribed strand to evaluate the efficiency of T<>T as a transcriptional block *in vitro*. Fig. 2*A* shows the results of transcription experiments conducted using 137-mers with or without a thymine photodimer. With the dimer-free template transcription continues to the end of the fragment generating transcripts 89 or 90 nucleotides long (see below). When the dimer is present in the transcribed strand transcription terminates at the dimer producing a doublet (see below). Within the sensitivity of our assay (which would detect 0.5–1.0% of the radioactivity of the full length transcripts) we conclude that RNAP did not transcribe past the T<>T. To learn where transcription actually begins and ends with respect to the photodimer, the bands in the doublet which represent truncated transcripts were indi-

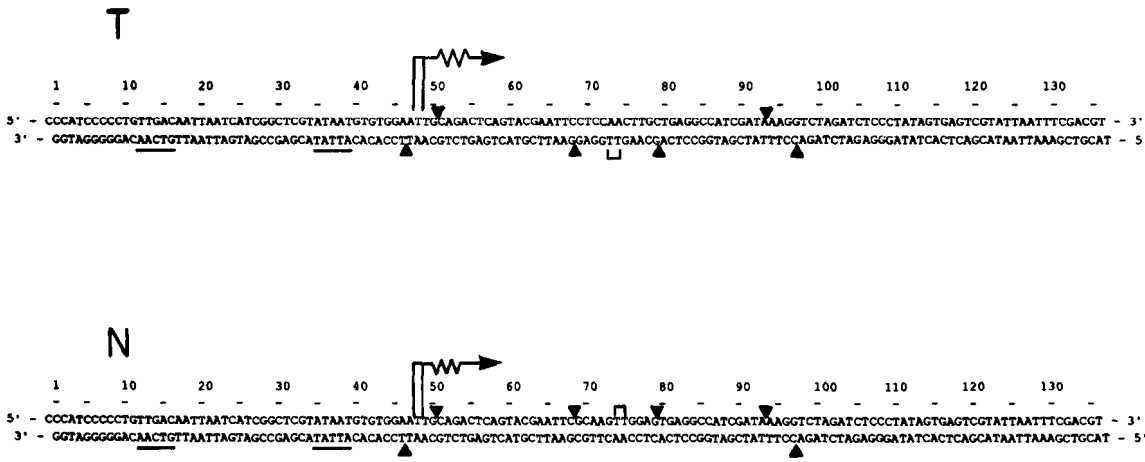

FIG. 1. **Synthetic transcription-repair substrate.** The 137-mers contain the *tac* promoter (−35 and −10 sequences *underlined*) and a thymine dimer (*T<>T*) downstream from the transcriptional initiation sites (*positions 48* and *49*) in the template (*T*) and the nontemplate (*N*) strands. The duplexes were constructed by ligating eight oligonucleotides whose boundaries are indicated by *triangles*. Transcription starts with UTP at positions 48 or 49 and proceeds in the direction indicated by the *arrow*.

vidually excised from the gel, purified, subjected to sequence-specific degradation reactions, and analyzed on an 11% polyacrylamide sequencing gel (Fig. 2*B*). Both bands from the doublet had the same sequences up to their 3′ ends which terminated with a base (see Fig. 2, legend) inserted opposite the 3′ T of the photodimer. The length heterogeneity of the truncated (and full length) transcripts must arise from differences at their 5′ ends, specifically from initiation at either the 9th or the 10th nucleotide 3′ to the −10 sequence. The latter is deduced from the DNA sequence (Fig. 1), the fact that the transcripts were end-labeled with UTP (and could not be end-labeled with ATP), and analysis of sequencing reactions on 20% gels (not shown) to obtain the sequence of the 5′ ends.

When the T<>T was in the nontranscribed strand it did not cause pausing by RNAP or premature termination of the transcript (Fig. 2*A*, *lane 8*). This result is consistent with the finding of Shi *et al.* (1987) that a psoralen-thymine monoadduct in the nontranscribed strand did not block transcription.

Stalling of RNAP at the Thymine Photodimer—To investigate the formation of stable RNAP-DNA-T<>T complexes at the lesion site we conducted DNase I footprinting experiments using the substrate with a T<>T in the template-strand. Incubation of RNAP with the substrate in the absence of rNTPs led to the formation of a stable initiation complex as evidenced by a standard RNAP footprint over the promoter region (Fig. 3, *lanes 2–5*). When rNTPs were included in the reaction mixture the RNAP footprint was no longer at the promoter, rather it was displaced in the 3′ direction, covering an area which includes the thymine photodimer (Fig. 3, *lanes 6–9*). Thus, as was found with a psoralen cross-link in transcribed DNA (Shi *et al.*, 1987), RNAP makes a stable elongation complex at the site of a T<>T in the template.

Effect of Transcription on Excision of T<>T from the Template and Nontemplate Strands by (A)BC Excinuclease—The data presented so far indicate that a T<>T in the transcribed strand blocks transcription resulting in a truncated transcript and a stable elongation complex. Does this complex facilitate the excision of T<>T by (A)BC excinuclease? (A)BC excinuclease hydrolyzes primarily the 8th phosphodiester bond 5′ and the 5th phosphodiester bond 3′ to a T<>T (Sancar and Rupp, 1983). The 137-mers N and T in Fig. 1 (dimer in the nontemplate and template strands, respectively) were labeled with ^{32}P at the 5′ end of the damaged strand and digested with ABC excinuclease. The end-labeled digestion product of N runs as a 67-mer on a denaturing gel and digestion of T gives a 56-mer (Fig. 4*A*). The T substrate is digested with somewhat higher efficiency than N, due to a moderate effect of neighboring sequences on enzyme activity. Incision of both substrates is inhibited at high concentrations of RNAP (Fig. 4*A*, *lanes 8* and *9*) due to nonspecific binding of RNAP to DNA (Fig. 3, *lanes 4* and *5*). When the reaction mixtures contained RNAP plus all four rNTPs, the incision of the transcribed strand was inhibited to a greater extent than incision of the nontranscribed strand. The optimal RNAP concentration (13 nM) for producing an elongation complex footprint (Fig. 3, *lanes 4* and *8*) drastically inhibited the excision of T<>T from the transcribed strand (Fig. 4*A*, compare *lanes 8* and *18*). The selective inhibition of incision of T<>T in the transcribed strand under transcription conditions was also observed when we measured the kinetics of incision (Fig. 4*B*). Thus, the data shows that RNAP stalled at a T<>T does not stimulate but actually inhibits the excision of the photoproduct by the excision nuclease.

The selective inhibition of incision of the template lesion was unexpected, yet it was consistently observed under conditions in which stimulation of incision by DNA photolyase was readily demonstrated (Fig. 4). Nevertheless we further examined the possible stimulation of incision of the template dimer by transcription. We reasoned that a low concentration of RNA polymerase might have a subtle enhancing effect on the kinetics of incision that we had not detected. However, the results obtained with the T substrate shown in Fig. 5 indicate that 0.7 nM RNAP did not stimulate incision kinetics while photolyase clearly did. Similarly, varied orders of addition of RNAP and (A)BC excinuclease failed to produce stimulation of incision (Fig. 2*A* and data not shown).

Photolyase as a Possible Coupling Factor—It has been suggested (Patterson and Chu, 1989) that photolyases function as auxiliary proteins which target lesions for repair by nucleotide excision. Therefore, we conducted further experiments to examine whether more stimulation could be achieved when both RNAP and *E. coli* DNA photolyase were present in the reaction mixture compared to photolyase alone. Addition of photolyase to a preformed elongation complex fails to relieve the inhibitory effect of RNAP and addition of RNAP to photolyase-T<>T complexes interferes with the stimulatory

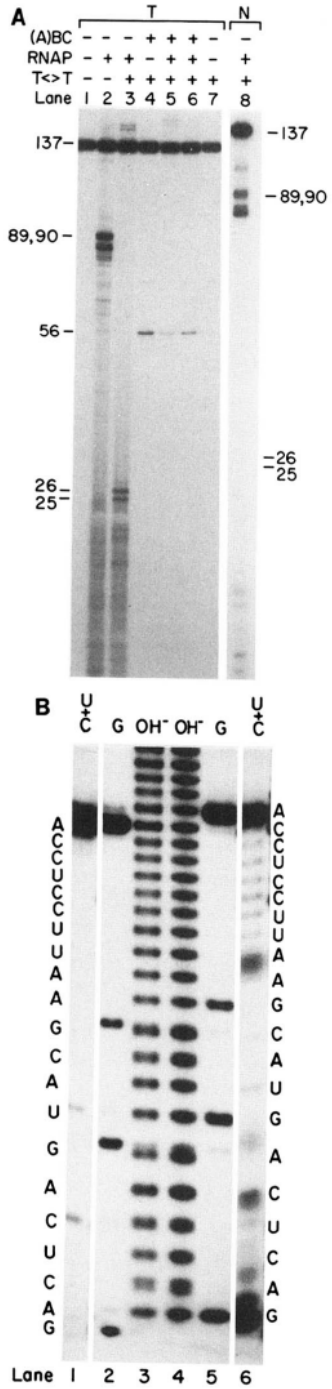

FIG. 2. *A*, effect of T<>T in the template strand on transcription. 5′ terminally labeled synthetic 137-mer ("T" in Fig. 1 labeled at the 5′ end of the strand containing the central 11-mer) without (*lanes 1* and *2*) or with T<>T (*lanes 3–7*) was used as substrate for RNAP and/or (A)BC excinuclease as indicated and the reaction products were analyzed on an 8% polyacrylamide sequencing gel. Transcripts were labeled at the 5′ end with [α-^{32}P]UTP. The positions of the full length DNA fragment (*137*), the DNA band generated by incision 7 bases 5′ to T<>T (*56*) and the full length transcripts (*89* and *90*) and truncated transcripts (*25* and *26*) are marked. In *lanes 4–6* the DNA was digested for 8 min with (A)BC excinuclease in the presence of rNTPs. In *lane 5* the DNA was preincubated with RNAP for 5 min before addition of (A)BC excision nuclease. In *lane 6*, the DNA was preincubated with UvrA and UvrB for 5 min before adding RNAP and UvrC. *Lane 8* is from a separate experiment in which the 137-mer containing T<>T in the nontemplate strand ("*N*" in Fig. 1) was transcribed. *B*, the transcription stopsite in relation to the dimer. 5′-end-labeled 25- and 26-base transcripts (*lanes 1–2* and *5–6*, respectively) formed in the presence of T<>T (as in *A*) were excised separately from gels, purified, and sequenced by using the enzymatic method of Donis-Keller *et al.* (1977). The RNA ladders in *lanes 3* and *4* were generated from alkaline hydrolysis of full length transcripts. Note that even though our data shows, unambiguously, that a nucleotide is inserted across from the 3′ T residue, we have not actually demonstrated that the inserted nucleotide is dAMP.

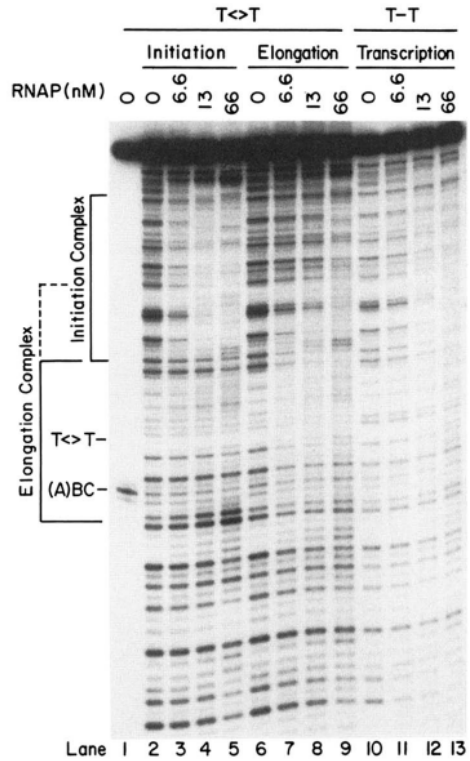

FIG. 3. **DNase I footprint of RNAP initiation and elongation complexes on the 137-mer with T<>T in the transcribed strand.** In *lane 1* the 5′ terminally labeled DNA substrate was treated with (A)BC excinuclease. The other lanes contain DNA that was incubated with RNAP for 10 min and then digested with DNase I. Samples in *lanes 2–5* contained ATP and UTP whereas the samples in *lanes 6–9* contained all four rNTPs. The footprints of RNAP initiation and elongation complexes are *bracketed*, and the region of possible overlap of the two footprints is indicated with a *broken line*. No discernible footprint is evident on the nondimer (*T-T*) template (*lanes 10–13*); however, there is an overall protection of the area 3′ to the promoter presumably because of transcription. The 5′ incision site of (A)BC excinuclease is indicated by (A)BC.

effect of photolyase (data not shown) suggesting that photolyase does not function as a coupling factor.

Transcription and Repair of Supercoiled, UV-irradiated DNA

Transcription causes drastic topological changes in DNA (Gamper and Hearst, 1982; Tsao *et al.*, 1989). It is conceivable that these topological features of transcribed DNA would be associated with the enhanced rate of repair observed *in vivo*. Along these lines Pu *et al.* (1989) recently reported that the rate of incision by (A)BC excinuclease of *N*-methylmitomycin cross-linked DNA was about 200-fold higher in supercoiled molecules than with relaxed molecules.

To investigate the effect of transcription on repair of superhelical DNA we used pDR3274, which was modified by irradiation with 254-nm light. Repair was measured as the UV-dependent formation of [α-^{32}P]dGTP-labeled repair patches synthesized by the combined action of ABC excinuclease, DNA *Pol*I, and DNA ligase. pDR3274 was selected because it contains a very strongly transcribed *UvrC* gene (Mulligan *et al.*, 1985; Thomas *et al.*, 1985) and the other genes are either inactive (*cam*) or weakly transcribed (*tet*,

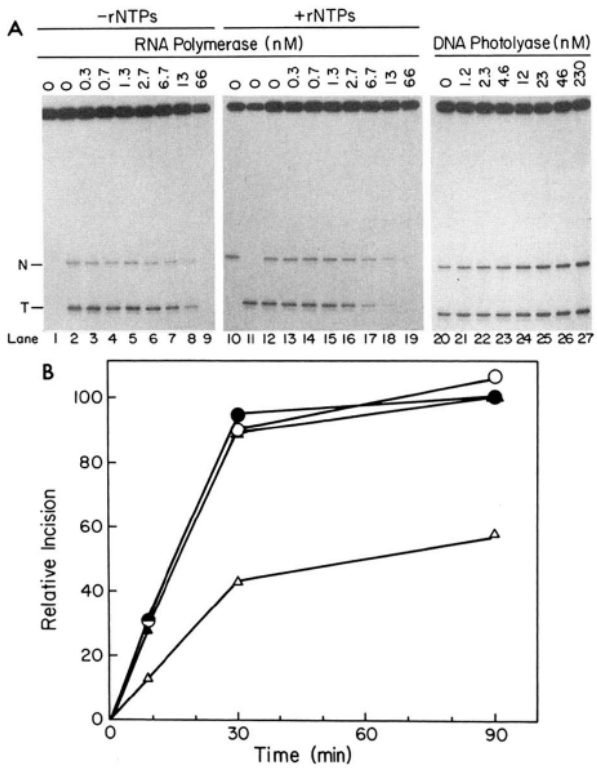

FIG. 4. *A*, effect of RNAP and DNA photolyase on removal of T<>T by (A)BC excinuclease. A mixture of 137-mer N and T substrates (each 5′-end-labeled on the strand containing the central 11-mer (Fig. 1), was preincubated in transcription buffer for 10 min with the indicated concentrations of RNAP to form either the RNAP-promoter (*−rNTP*) or the elongation complex (*+rNTP*), or photolyase to form the stable photolyase enzyme-substrate complex. Then (A)BC excinuclease was added and incubation continued for another 15 min. The reaction products were analyzed on 8% polyacrylamide sequencing gels. The location of the fragments generated by incision 5′ to T<>T in the nontranscribed (*N*) or transcribed (*T*) strands by (A)BC excinuclease are indicated. In *lanes 10* and *11*, the N and T substrates were individually digested with (A)BC excinuclease, and in *lane 1* the mixture of N and T was not digested. The different panels are from separate experiments which employed slightly different ratios of the N and T substrates. *B*, kinetics of incision of the transcribed and nontranscribed strands by (A)BC excinuclease. The reaction mixture contained 6.6 nM RNAP. Repair reactions were carried out for the indicated times, reaction products were analyzed on 11% polyacrylamide sequencing gels, and the relative amounts of incision were quantified by scanning densitometry of autoradiograms. The level of incision for each substrate is expressed relative to the level of incision obtained in the absence of rNTPs, which was given a value of 100. *Triangles*, T<>T in the transcribed strand; *circles*, T<>T in the nontranscribed strand; *closed symbols*, nontranscribing conditions (−rNTPs); *open symbols*, plus rNTPs. Data points are the mean of three separate experiments, standard errors ranged from 2 to 8 units of relative incision.

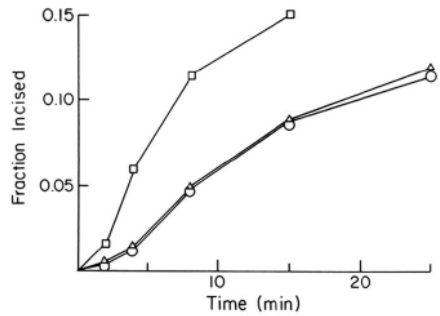

FIG. 5. **Effect of an RNAP elongation complex or a photolyase enzyme-substrate complex on the rate of incision 5′ to T<>T by (A)BC excinuclease.** The 137-mer (T<>T in the template strand) was preincubated with no additions (○), 0.7 nM RNAP (△), or 30 nM DNA photolyase (□) in transcription buffer with rNTPs, then (A)BC excinuclease was added and incubation continued at 37 °C. At the indicated times, samples were taken and the reactions were stopped. Samples were precipitated with ethanol, reaction products were separated on 8% polyacrylamide sequencing gels, and the bands corresponding to full length and incised 137-mer were quantified by scanning densitometry. The fraction incised was calculated as the amount incised divided by the total DNA (incised plus undigested) for each lane.

Bertrand-Burggraff *et al.*, 1984). By separating the *UvrC* gene from silent regions of the plasmid after the repair synthesis reactions, we could compare repair in the *UrvC* gene with repair in transcriptionally inactive regions.

We first examined transcription of pDR3274 by examining transcripts that were internally labeled with [α-^{32}P]UTP on a 6.5% sequencing gel (data not shown). pDR3274 has three transcriptional units: *tac-UvrC* (1800 bp), *tet* (1200 bp), and RNA-I (108 bp). With supercoiled template we established that transcription of this plasmid generates primarily *tac-UvrC* mRNA. We optimized the transcription conditions and found that with 200 nM RNAP the rate of transcription was about 3600 nucleotides plasmid^{-1} min^{-1} which corresponds to approximately two transcripts min^{-1} assuming that all the RNA synthesis was from the *tac* promoter. This value is reasonably close to the 2.3 transcripts min^{-1} for the *lac* operon *in vivo* under inducing conditions (Lewin, 1987).

We used UV-irradiated pDR3274 in our transcription-repair experiments. Production of full length *tac-uvrC* transcripts is inhibited in a dose-dependent manner, the transcription level being reduced to approximately e^{-1} (data not shown) at a dose (30 J/m^2) which is expected to produce about one dimer per strand of the transcription unit. We carried out repair synthesis with DNA irradiated with 75 Jm^{-2}, which produces about four dimers per *uvrC* gene. The reaction mixtures contained a full complement of transcription, repair, and resynthesis proteins in addition to the necessary cofactors and [α-^{32}P]dCTP as a tracer to measure repair synthesis. We used reaction-limiting amounts of the (A)BC excinuclease subunits so that repair synthesis was clearly detectable above the background yet far below saturating in order to detect any stimulatory effect of transcription. Following the transcription-repair reaction, the plasmid was digested with *Eco*RI, *Hin*dIII, and *Bgl*II to produce: (*a*) an ~1.2-kb *Eco*RI-*Hin*dIII fragment which is not transcribed; (*b*) an ~2.1-kb *Eco*RI-*Bgl*II fragment containing the strongly transcribed *UvrC* gene; and (*c*) an ~2.9-kb *Bgl*II-*Hin*dIII fragment containing the *tet* gene and the origin of replication.

The fragments were separated on agarose gels which were stained, photographed, and autoradiographed. The results (Fig. 6 and Table I) indicate that the transcribed region of the plasmid is not repaired more efficiently than the nontranscribed regions. With similar concentrations of UV-irradiated DNA and Uvr proteins, the stimulatory effect of photolyase on repair synthesis was easily detectable (Sibghat-Ullah and Sancar, 1990). We considered the possibility that the topological consequences of transcription might be transmitted through the whole plasmid resulting in enhanced repair throughout, thus obscuring gene-specific repair. However, when a comparison is made between repair of all three fragments with or without transcription, no difference is seen (Fig. 6 and Table I). In fact, considering the results obtained with linear DNA one might expect preferential inhibition of repair synthesis of the transcribed sequence when compared to either of the other fragments. While there appears to be a trend in that direction (Table I) the differences are not statistically significant ($p < 0.005$, Student's t tests). A plau-

sible explanation for this lack of inhibition is that the inhibitory effect is diluted out because half of the photoproducts are in the nontranscribed strand (repair not inhibited) and stalling of RNAP at the first photoproduct in the template would not inhibit the repair of photoproducts downstream. Attempts to demonstrate an inhibitory effect with DNA containing on the average one T<>T per *uvrC* were unsuccessful due to the small differences in repair synthesis when comparing singly adducted *versus* undamaged plasmids (data not shown). Also, higher RNAP concentrations than the one used in these experiments actually inhibited repair synthesis globally (data not shown).

DISCUSSION

Gene- and strand-specific repair are fascinating concepts both biologically and biochemically. From a biological standpoint, for example, it has been suggested (Bohr *et al.*, 1985) that preferential repair of actively transcribed genes may explain the rodent cell paradox: rodent cells which are as resistant to UV as human cells remove only 20% of cyclobutane pyrimidine dimers from bulk DNA in 24 h compared to human cells which remove more than 80% within the same period. However, if the comparison is made at the gene level both cell types remove 80% of the photodimers. The implications are that cells need only to repair genes which control

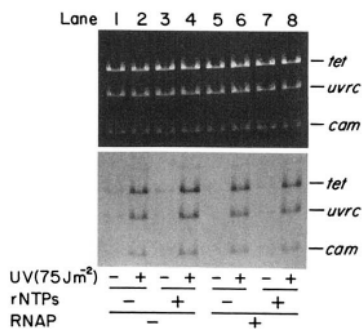

FIG. 6. **Effect of transcription on repair of superhelical plasmid by (A)BC excinuclease as measured by repair synthesis.** Repair synthesis reactions (with DNA polymerase I and DNA ligase) were conducted with undamaged or damaged (75 Jm^{-2}) pDR3274, rNTPs, and RNAP (130 nM) as indicated. The incubation with (A)BC excinuclease and DNA *polI* plus ligase was for 8 min with 2–8 μCi [α-^{32}P]dCTP per reaction as the tracer. Following transcription-repair the DNA was digested with *Eco*RI, *Bgl*II, and *Hind*III and separated on a 1.2% agarose gel. *Top*, photograph of ethidium bromide-stained gel; *bottom*, autoradiograph of the same gel. The bands which carry the strongly transcribed *uvrC*, weakly transcribed *tet*, and nontranscribed (promoterless) *cam* gene are indicated.

essential functions and that photodimers do not constitute an absolute block to replication and thus can be tolerated. Evidence exists for tolerance mechanisms at the replicational level in both pro- and eucaryotes (Piette and Hearst, 1983; Yang *et al.*, 1982; Vos and Hanawalt, 1987) and such a model makes teleological sense.

In contrast, a comprehensive biochemical model which takes into account the known properties of the transcription apparatus and the nucleotide excision repair system has been difficult to formulate. Three general concepts have been considered: (*a*) the open conformation of chromatin in transcribed regions may increase accessibility to the repair enzyme; (*b*) RNAP stalled at a photodimer facilitates the binding and rate of repair by the subunits of the excision repair enzyme in a manner analogous to that of *E. coli* photolyase (Sancar *et al.*, 1984) and perhaps yeast photolyase (Sancar and Smith, 1989); (*c*) the unique topology created at the transcription site by overwinding of DNA in front of the transcription complex and underwinding behind it (Tsao *et al.*, 1989) generates a structure with higher affinity for the repair enzyme. Although the open chromatin structure in both pro- and eucaryotes in transcribed regions may make DNA damage more accessible to repair enzymes, it cannot account for the strand selectivity of the excision nuclease unless the template strand is in some way made more accessible than the nontemplate strand in the open chromatin structure. Smerdon and Thoma (1990) suggested that when RNAP stalls at the site of a lesion in the transcribed strand, the transcribed region remains in the "open configuration" for longer periods and thus allows more time for the excision nuclease to repair lesions in the template strand. The work reported here indicates that *in vitro*, RNAP stalled at a lesion does not itself facilitate repair and may indeed impede repair. We show that global effects produced by the transcription of superhelical DNA do not change the rate of repair by (A)BC excinuclease in transcribed sequences. Admittedly, our *in vitro* experiments cannot faithfully reproduce all the topological changes one would expect to observe *in vivo*; therefore this issue is not totally resolved. An alternative is that there must be a coupling factor missing in our *in vitro* system which links transcription to repair. A strong candidate for such a factor in *E. coli* is photolyase which is known to stimulate excision repair on its own. However, we found that a greater stimulatory effect by photolyase in the presence of RNAP did not occur. We must therefore conclude that neither photolyase nor its possible mammalian analog (Patterson and Chu, 1989) are responsible for coupling repair to transcription.

In this paper we present the first unambiguous evidence

TABLE I

Effect of transcription on repair synthesis in defined regions of pDR3274

Values represent damage-specific cpm incorporated and were derived from each +UV/−UV treatment pair such as those shown in Fig. 6 by subtracting the cpm in the unirradiated DNA band (which ranged from 13 in *tet* to 2 in *cam* in experiment 1; 32 in *tet* to 0 in *cam* in experiment 2; and 66 in *tet* to 8 in *cam* in experiment 3) from the cpm in the UV-irradiated DNA band.

Fragment	Repair synthesis experiments						+RNAP/−RNAP
	−RNA polymerase			+RNA polymerase			
	1	2	3	1	2	3	
	cpm						
tet	86(30)[a]	61(21)	212(73)	71(25)	79(27)	232(80)	1.07 ± 0.13
uvrC	61(29)	36(17)	262(125)	37(18)	50(24)	227(108)	0.97 ± 0.23
cam	28(24)	18(15)	129(107)	29(24)	29(24)	91(76)	1.10 ± 0.26

[a] Values in parentheses are cpm per kilobase pair (*cam*, 1.2; *uvrC*, 2.1; *tet*, 2.9 kbp). +RNAP/−RNAP represents the ratios of cpm per kilobase pair; the means and standard errors of ratios obtained in the three experiments are given.

that a thymine dimer is a strong block to transcription; this has long been inferred but never proven conclusively in either pro- or eucaryotes (Sauerbier and Hercules, 1978). We show that RNAP transcribes up to and including the nucleotide across from the 3′ T of the dimer in about 90% of cases (Fig. 2) and stops at the nucleotide across from the 5′ T of the dimer in 10% of the molecules (data not shown). For comparison, Shi *et al.* (1988) found that with psoralen-cross-linked and -monoadducted templates, *E. coli* RNAP stopped at the last base before the adducted T residue. Transcription up to and including the 3′ T of the dimer is one base further than the stopsite of DNA polymerase I at a dimer reported by Moore and Strauss (1978), although a more recent report has shown that *Pol*I can insert dAMP across from the 3′ T (Taylor and O'Day, 1990).

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