Transcription Arrest at a Lesion in the Transcribed DNA Strand * 

In Vitro Is Not Affected by a Nearby Lesion in the Opposite Strand

Cis-syn cyclobutane pyrimidine dimers (CPDs) are the most frequently formed lesions in UV-irradiated DNA. CPDs are repaired by the nucleotide excision repair pathway. Additionally, they are subject to transcription-coupled DNA repair. In the general model for transcription-coupled DNA repair, an RNA polymerase arrested at a lesion on the transcribed DNA strand facilitates repair by recruiting the repair machinery to the site of the lesion. Consistent with this model, transcription experiments in vitro have shown that CPDs in the transcribed DNA strand interfere with the translocation of polynucleotide and eukaryotic RNA polymerases. Here, we study the behavior of RNA polymerase when transcribing a template that contains two closely spaced lesions, one on each DNA strand. Similar DNA templates containing a single CPD on either the transcribed or the nontranscribed strand were used as controls. Using an in vitro transcription system with purified T7 RNA polymerase (T7 RNAP) or rat liver RNAP II, we characterized transcript length and efficiency of transcription in vitro. We also tested the sensitivity of the arrested RNAP II-DNA-RNA ternary complex, at a CPD in the transcribed strand, to transcription factor TFIIS. The presence of a nearby CPD in the nontranscribed strand did not affect the behavior of either RNA polymerase nor did it affect the reverse translocation ability of the RNAP II-arrested complex. Our results additionally indicate that the sequence context of a CPD affects the efficiency of T7 RNAP arrest more significantly than that of RNAP II.

It has been nearly two decades since the discovery that in UV-irradiated cells of eukaryotes and prokaryotes the transcribed strand of an active gene is repaired more rapidly than the nontranscribed strand (1, 2). However, the mechanistic details of this phenomenon, termed transcription-coupled repair (TCR),3 are still not fully understood. The general model for the mechanism of TCR postulates that an RNA polymerase stalled at a lesion is the signal for recruitment of the repair proteins and initiation of excision repair (3). In the case of UV-induced DNA lesions, such as the most frequently formed cis-syn CPDs, the specific repair process requires that an oligonucleotide containing the damaged site is excised from the DNA, the gap created is closed by repair replication, and finally the repair patch is ligated to the contiguous DNA (reviewed in Refs. 4 and 5). An active RNA polymerase is a prerequisite for TCR (6–8). However, the arrested polymerase must then be displaced so that the lesion becomes accessible to the repair machinery (3). In Escherichia coli, a transcription-repair coupling factor (Mfd) has been shown to displace the stalled polymerase and facilitate TCR (9). In eukaryotes, however, it is still not clear how the arrested polymerase signals the assembly of the repair machinery at the site of damage, nor is it clear how the polymerase is displaced to allow access of the repair proteins to the damaged site. Despite the evidence that incomplete transcripts, because of RNA polymerase arrest at the site of damage, are released in E. coli (9), such a mechanism has not been confirmed for eukaryotes. Especially in the case of very large transcription units such as the 2.5-megabase dystrophin gene, it would seem energetically wasteful for the cell to abort transcription and release the incomplete nascent transcript. One of the models for the mechanism of TCR in higher organisms proposes that the polymerase might back up, allowing accessibility of repair enzymes to the damaged site, and then transcription could resume once the lesion is repaired (3, 10, 11). The transcription factor TFIIS, which facilitates transcription through natural pause sites, may participate in this mechanism. TFIIS is widely distributed in eukaryotes and enables RNAP II to resume transcription after arrest at pause sites and to complete long RNA transcripts (12). Evidence from experiments in vitro suggests that TFIIS binds to an arrested polymerase and activates its cryptic 3′ → 5′ endonuclease activity, resulting in the hydrolysis of the nascent transcript at internal phosphodiester bonds. It is believed that the 3′-hydroxyl group of the resulting shorter transcript is then correctly placed in the catalytic site of the enzyme such that nucleotide polymerization can resume (13).

In vitro transcription systems with DNA templates containing different types of lesions have proved very useful for demonstrating that several types of lesions in the transcribed DNA strand can block transcription by different RNA polymerases (reviewed in Ref. 11). These findings are in agreement with the proposed model for TCR that requires an arrested RNA polymerase to signal the recruitment of the repair enzymes to the damaged site (14). CPDs in the transcribed strand block transcription elongation by mammalian RNAP II (10, 11). They also block transcription elongation by T7 RNAP although less efficiently (15). When present on the nontranscribed strand, CPDs do not impede transcription elongation by RNAP II (reviewed in Ref. 11). Depending upon the sequence context, however, they can affect the rate of elongation or stalling of the polymerase (16). In this study we examined the behavior of T7 RNAP...
and mammalian RNAP II when transcribing a template that contains two closely spaced lesions, one on each strand, to investigate their possible combinatorial effect upon transcription elongation. For this purpose, we have employed an in vitro transcription system with T7 RNAP or RNAP II from rat liver, and a set of DNA templates that contain no CPDs, a CPD either on the transcribed or on the nontranscribed strand, or two CPDs, one on each strand. All lesions were positioned downstream of the prokaryotic T7 promoter or the eukaryotic adenovirus major late promoter (AdMLP). We found that two lesions were present in the DNA template, transcription arrest of both T7 RNAP and RNAP II occurred at the same location as in the template with a single CPD in the transcribed strand, indicating that the CPD in the nontranscribed strand had no effect on the response of either polymerase.

It was demonstrated previously that when RNAP II from rat liver is stalled at a CPD in vitro, addition of TFIIIS caused cleavage of the nascent transcript and reverse translocation of the polymerase, up to 35 nucleotides upstream of the lesion without disruption of the ternary complex (17). This facilitated repair of the CPD when a small repair enzyme, photolyase, was added into the reaction; subsequent resumption of transcription past the repaired site was documented. We tested the effect of TFIIIS on the behavior of RNAP II by transcribing the template with the two closely opposed lesions and observed the same cleavage pattern with the template carrying a single CPD on the transcribed strand.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 DNA ligase, and T7 RNAP were purchased from New England Biolabs. T4 polynucleotide kinase was purchased from Amersham Biosciences. RNAP II, transcription initiation factors, and TFIIIS purified from rat liver or recombinant sources, as described previously, were obtained from Dr. Daniel Reines (18). Highly purified NTPs were purchased from Amersham Biosciences. D44 IgG anti-RNA antibodies (19) were purified from rodent ascites fluid as described (18) and formalin-fixed Staphylococcus aureus was obtained from Calbiochem. Custom made DNA oligonucleotides were purchased from Qiagen. Oligonucleotides containing site-specific CPDs, constructed as described previously (20, 21), were obtained from Dr. John Stephen Taylor (Washington University, St. Louis, MO).

Preparation of Templates for in Vitro Transcription with T7 RNA Polymerase—Four DNA templates, 139 bp long, containing the T7 promoter and (a) no CPDs, (b) a CPD on the transcribed strand, (c) a CPD on the nontranscribed strand, or (d) two CPDs, one on each DNA strand and 29 nucleotides apart, were constructed (Fig. 1A). The templates were constructed by annealing and ligating together nine oligonucleotides. A 54-mer (5′-TACGTCGAAATTAATACGACTCACTATAGGG-GATCTAGACATCATGGCGACCA-3′), a 15-mer (5′-TACGTCGAAATTAATACGACTCACTATAGGG3′), a 12-mer (5′-CACCCTGCTCG- TGGAG-3′), and a 58-mer (5′-CTAGTGGAAATTAATACGACTCACTATAGGGGATCTAGACATCATGGCGACCA-3′) were used for the nontranscribed strand, and one on each DNA strand and 29 nucleotides apart, were constructed (Fig. 1A). The templates were constructed by annealing and ligating together nine oligonucleotides. 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Effect of Closely Spaced DNA Lesions on Transcription

RESULTS

Effect of Closely Spaced CPDs, One on Each DNA Strand, on Transcription Elongation by T7 RNAP—Four DNA templates (Fig. 1A), 139 bp long, were prepared to be used in an in vitro transcription system with RNA polymerase from bacteriophage T7. They were assembled by annealing and ligating together nine oligonucleotides, two of which, one on each strand, did or did not contain a thymine dimer. The final ligation products resulted in four templates with the T7 promoter and (i) no CPDs, (ii) a CPD in the nontranscribed strand, 52 nucleotides downstream of the transcription start site, or finally (iv) one CPD present on each strand. The presence of the lesions at the predicted site was confirmed by digestion with T4 endonuclease V (22, 23), which nicks at the site of a CPD (data not shown).

Full-length run-off transcription from the control template with no CPDs resulted in an RNA transcript of 112 ribonucleotides (Fig. 2, lane 2). The presence of a CPD in the nontranscribed strand had no effect on transcription by T7 RNAP and resulted in transcripts that were also 112 nucleotides long (Fig. 2, lane 3). When the two templates with the lesion in the transcribed strand were used for transcription, a shorter transcript, 82 ribonucleotides long, predominated (Fig. 2, lanes 4 and 5). This transcript corresponded to the sequence length between the transcription start site and the lesion. However, in contrast with the results with mammalian RNAP II, where the lesion on the transcribed strand is a complete block for the eukaryotic polymerase (Refs. 10 and 11 and this study), about 45% of the T7 RNA polymerase molecules were able to bypass the lesion and produce full-length run-off transcripts (Fig. 2, lane 4, Fig. 3, and Fig. 4A). Similar results were observed when the second CPD was present in the nontranscribed strand (Fig. 4B).
Effect of Closely Spaced DNA Lesions on Transcription

FIG. 2. Transcription elongation by T7 RNAP using damaged templates. The four different templates were transcribed in vitro. –, template carrying no CPD (lane 2); NTS, a CPD in the nontranscribed strand (lane 3); TS, a CPD in the transcribed strand (lane 4); NTS + TS, two CPDs, one on each strand (lane 5). Elongation was allowed to proceed for 30 min before the radiolabeled transcripts were purified and electrophoresed through an 8% denaturing polyacrylamide gel. L (lane 1), 10-bp DNA ladder; RO, full-length run-off transcript; TT with caret (†), truncated transcripts due to arrested T7 RNAP at the site of the CPD.

2, lane 5, and Fig. 3, Fig. 4B). To determine whether there were any differences in the rate of accumulation of the truncated and full-length transcripts we measured the time course of transcription after 15 s, 30 s, 45 s, 1 min, 2.5 min, and 5 min after all RNA molecules were synchronized at position 6 (see “Experimental Procedures”) and heparin and ribonucleotides were added into the reaction mixture (Figs. 3 and 4). We observed that at earlier time points, there is a higher rate of accumulation of the truncated transcripts compared with full-length transcripts. However, at later time points, some of the initially paused polymerases were able to bypass the lesion and give rise to full-length transcripts (Figs. 3 and 4). The presence of the second CPD on the nontranscribed strand did not affect the relative rate of accumulation of the two transcripts.

Effect of Closely Spaced CPDs, One on Each DNA Strand, on Transcription Elongation by Mammalian RNAP II—Four different DNA templates, 391 bp long, were also prepared to be used as templates in an in vitro transcription system with RNAP II and transcription factors purified from rat liver. All four templates have the identical nucleotide sequence and contain the adenovirus major late promoter and a G-less cassette that allows synchronization of the transcribing polymerases and radioactive labeling of the transcript before the addition of GTP into the transcription reaction. The first template contained no CPDs. The second template, contained a CPD positioned in the nontranscribed strand, 178 bases downstream of the transcription initiation site. The third template contained a CPD in the transcribed strand, 206 bases downstream of the transcription initiation site, and the forth one contained both lesions of the two previous templates (Fig. 1B).

The same full-length run-off transcript was observed when either the undamaged control template or the template containing a CPD in the nontranscribed strand was used as a template in the transcription reaction (Fig. 5, lanes 2 and 3). When the CPD was present in the transcribed strand, a transcript shorter than the full-length run-off transcript was produced (Fig. 5, lane 4). The length of this shorter transcript corresponded to that synthesized by an RNA polymerase molecule that had translocated up to the site of the lesion. The same result was obtained when the DNA contained a second CPD in the nontranscribed strand, located 26 bases upstream of that in the transcribed strand (Fig. 5, lane 5).

Effect of Closely Spaced CPDs, One on Each DNA Strand, on Transcript Cleavage by Elongation Factor TFIIS—We also wanted to investigate whether the presence of the second lesion in the nontranscribed strand had any effect on transcript cleavage by the transcription elongation factor TFIIS. For this purpose, the template with the lesion in the transcribed strand and the template with the two lesions were used again as templates for in vitro transcription with eukaryotic RNAP II. The stalled RNAP II-DNA-RNA ternary complexes were purified by immunoprecipitation and then treated with TFIIS. In both cases (Fig. 6) treatment with the elongation factor caused similar cleavage patterns of the nascent transcript, indicating that the presence of the second lesion in the nontranscribed strand did not affect the reverse translocation ability of the arrested RNAP II ternary complex.

DISCUSSION

When the phenomenon of TCR was discovered (1), it was hypothesized that an arrested RNA polymerase was required to signal the assembly of the repair enzymes at the site of the lesion and elicit repair. Since then, a number of in vitro transcription systems using the prokaryotic T7 and E. coli RNA polymerases or eukaryotic RNAP II from different species have
proven very useful in testing the ability of various DNA lesions to block transcription elongation. We now know that most of the well studied DNA lesions subject to TCR present a block to translocation by both prokaryotic and eukaryotic RNA polymerases when present in the transcribed strand, whereas they fail to do so when present in the nontranscribed strand (reviewed in Ref. 11). There are no previous reports on the possible combinatorial effect of closely spaced DNA lesions on transcription elongation. We have addressed the question of whether the presence of two closely spaced cyclobutane thymine dimers, one on each strand, on a DNA region transcribed either by mammalian RNAP II or prokaryotic T7 RNAP, alters the effect that each lesion would have by itself on the behavior of the elongating RNA polymerase.

In contrast to the multi-subunit eukaryotic RNA polymerases, T7 RNA polymerase is monomeric. Despite its small size and its limited structural homology with other RNA polymerases, however, it maintains all of the fundamental characteristic features of RNA polymerases, such as specific promoter recognition and initiation of transcription, abortive initiation, and conversion to a stable, elongating ternary complex (24). All of these characteristics, in combination with the fact that the highest resolution structural information available for RNA polymerases is that for T7 RNAP, make it a very useful system. Previous studies have investigated the effect of several types of lesions on transcription elongation by T7 RNAP. Psoralen monoadducts and diadducts (25), benz(a)pyrene (26, 27), benzo(c)phenanthrene (28), tetrahydrofuran, acetylaminofluorene, and aminofluorene (29), and thymine glycol (30, 31) block the elongating T7 RNAP when present in the transcribed strand, whereas aminofluorene (29) and cytosine arabinose do not block it (32).

Our results confirm that the predominant form of thymine dimers, the cis-syn photoproduct, creates a block for the elongating T7 RNAP when present in the transcribed strand. About 55% of the total transcripts produced when a CPD was present in the transcribed strand were shorter than the full-length run-off transcripts (Fig. 2, lane 4, Fig. 3, lane 7, and Fig. 4A). Smith and co-workers (15) have tested several of the lesions frequently induced at dipyrimidine sites by UV (4), including cis-syn and trans-syn (I and II) cyclobutane dimers and the 6–4-pyrimidine-pyrimidone photoproducts and their Dewar...
valence isomers. They found that all block transcription by T7 RNAP, but with very different efficiency (15). For the particular sequence tested, the trans-syn II photoproduct was the strongest block to transcription elongation, whereas the cis-syn dimer, contrary to our results, was only a very weak block. This result, in combination with ours, indicates that the sequence context around the site of the CPD can have a strong effect on its ability to block the elongating T7 RNAP. Similar results, revealing the effect of sequence context on the efficiency of blockage by the DNA lesion, have also been observed for thymine glycol (30, 31).

We observed that in addition to the predominant truncated transcript that corresponded to termination at the last nucleotide before the lesion, two more products, one and two nucleotides longer than the predominant one, were also formed. Similar transcripts have also been observed when T7 RNAP encountered a thymine glycol in the transcribed strand (31). It has been proposed that, at least for some types of lesions (26, 28), insertion of the correct ribonucleotide opposite the blocking adduct allows T7 RNAP to bypass the block and produce a full-length transcript. On the contrary, insertion of the wrong ribonucleotide may render the ternary DNA-RNA complex unstable and lead to dissociation and release of the truncated transcript. Depending upon the type of lesion, some noncomplementary ribonucleotides that are placed opposite the adduct can also permit translocation and allow bypass, however to a lesser extent. We speculate that the two truncated transcripts we observed were aborted products that incorporated the incorrect ribonucleotide opposite either the first or the second thymine of the thymine dimer, resulting in an unstable RNA-DNA hybrid that leads to the premature termination of transcription. Alternatively, the longer than expected transcripts could be because of incorporation of additional nucleotides by T7 RNAP during template slippage (33).

Interestingly, it has been shown that T7 RNAP can efficiently bypass one to five base gaps on the transcribed strand and even up to 24 bases, albeit with reduced efficiency (34), indicating that the unpaired nontranscribed strand is sufficient for bringing the downstream transcribed strand into the active site of the polymerase. These results revealed the importance of the nontranscribed strand in transcription elongation. The results from several other studies, however, indicated that benzo(a)pyrene (27), thymine glycol (31), 8-oxoguanine, tetrahydrofuran, aminofluorene, and acetylaminofluorene (29) lesions in the nontranscribed strand do not block T7 RNAP. Consistent with these results, we found that templates containing a CPD in the nontranscribed strand (but not in the transcribed strand) yielded the same transcription products as undamaged control templates. Thus, whatever the role of the nontranscribed strand in transcription elongation, lesions in the nontranscribed strand usually do not block the polymerase. With both templates we observed a small number of transcripts that were one nucleotide longer than the predominant full-length product. Other investigators have also reported T7 RNA-dependent transcripts one nucleotide longer or shorter than the predominant full-length product (15, 32, 33).

We also investigated the effect on transcription elongation of a second lesion present in the nontranscribed strand when a blocking lesion is already present in the transcribed strand (less than 30 nucleotides downstream). Our hypothesis was that the presence of two closely spaced lesions might create some conformational change in the DNA or in the interactions between the DNA template and elongating RNA polymerase, such that a lesion in the nontranscribed strand, which by itself does not affect the behavior of the transcribing polymerase, would do so when another blocking lesion is nearby in the transcribed strand. However, we found that an elongating T7 RNAP transcribed DNA templates carrying the two lesions with the same efficiency and similar degree of arrest as the template with only one lesion in the transcribed strand (Fig. 2, lane 5, Fig. 3, and Fig. 4B). These results indicate that transcription arrest most likely requires accessibility of the lesion to the active site of the elongating T7 RNAP. Even though the nontranscribed strand may play an important role during elongation, it is always outside the active site of the enzyme (35), which seems to allow some flexibility in the nucleotide structure required for undisrupted transcription.

In contrast to the small single-subunit T7 RNAP, eukaryotic RNAP II comprises 12 subunits and has a mass of about 600 kDa. It is highly conserved from yeast to mammals and is responsible for transcribing all eukaryotic mRNA. Its atomic structure has been solved at a 2.8 Å resolution (36). Lesions that have been shown to block an elongating RNAP II in vitro when present on the transcribed strand include cisplatin GG intrastrand and interstrand cross-link (37–39), N-acetoxy-acetylaminofluorene (40), psoralen monoadducts and interstrand cross-links (41), benzo(a)pyrene (42), and CPDs (10, 16, 17, 43).

We carried out experiments similar to those just described for T7 RNAP using purified mammalian RNAP II and transcription factors from rat liver. We confirmed that a CPD in the transcribed strand is a complete block for RNAP II in the sequence context that we used (Fig. 5). The same result was observed when a second CPD was present in the nontranscribed strand (Fig. 5). Thus, the behavior of an elongating RNAP II was not affected by the presence of a second lesion in the nontranscribed strand, nor did the presence of the lesion in the transcribed strand affect the behavior of the polymerase when it transcribed past the lesion in the nontranscribed strand. The undamaged control template and the one with a single CPD in the nontranscribed strand behaved in the same way (Fig. 5), confirming previous reports that a CPD in the nontranscribed strand does not affect the behavior of a transcribing RNAP II (10), unless it has an effect on the inherent structure of the DNA helix (16). In this case, when a thymine dimer was introduced into the nontranscribed strand of a naturally strong RNAP II transcription arrest site, it caused the arrest site to become weaker and only transiently arrested the polymerase. Similar effects have been observed with aminofluorene and N-acetoxy-acetylaminofluorene when present in the nontranscribed strand near the locus of a natural pause site (40). In these studies, even though the polymerase was not stalled by the lesion in the nontranscribed strand, natural pause site became stronger. Other lesions that have been tested for their effect on transcription elongation when present in the nontranscribed strand include benzo(a)pyrene (42), cisplatin GG interstrand cross-link (37), and thymine glycol (31). In all cases, and in agreement with what was observed for T7 RNAP, the lesion did not affect transcription in vitro by RNAP II.

It has been demonstrated that an arrested RNAP II at a CPD creates a stable complex (10, 43). When TFIIS was incubated with the arrested complexes, the RNA polymerase cleaved up to 35 nucleotides from the nascent RNA and backed up on the DNA while still maintaining a stable ternary complex that was capable of resuming transcription once the lesion was removed (17). To test whether the second CPD in the nontranscribed strand had any effect upon the reverse translocation ability of the complex arrested at the lesion in the transcribed strand, we compared transcript cleavage by the elongation factor TFIIS in the presence or absence of that second CPD (Fig. 6). If the presence of the second CPD led to the disruption of the stable
complex and caused the dissociation of the RNA polymerase, no shorter transcripts would be observed. In addition, because that second CPD is located within the DNA region where the polymerase backsteps in the presence of TFIIIS, we wanted to test whether its presence in any way obstructed the backtracking of the polymerase. Our results show that the presence of a second CPD on the nontranscribed strand does not affect the TFIIIS sensitivity of the arrested RNAP II complex (Fig. 6). Again, similar to the observations for T7 RNAP, these findings suggest that behavior of RNAP II is more likely to be affected when encountering altered DNA structures present on the transcribed strand that is accessible to the active site of the enzyme than when these structures are present on the nontranscribed strand.

In general, on the basis of the existing data on blockage of the RNA polymerases by different DNA adducts, it appears that T7 RNA polymerase has an increased ability to bypass lesions compared with the eukaryotic RNAP II. The crystal structures of these enzymes (35, 36) suggest that this could be attributed to the more "spacious" active site of T7 RNA polymerase (42). Based upon the fact that RNA polymerase arrest at a lesion is a complex and caused the dissociation of the RNA polymerase, no shorter transcripts would be observed. In addition, because that second CPD is located within the DNA region where the polymerase backsteps in the presence of TFIIIS, we wanted to test whether its presence in any way obstructed the backtracking of the polymerase. Our results show that the presence of a second CPD on the nontranscribed strand does not affect the TFIIIS sensitivity of the arrested RNAP II complex (Fig. 6). Again, similar to the observations for T7 RNAP, these findings suggest that behavior of RNAP II is more likely to be affected when encountering altered DNA structures present on the transcribed strand that is accessible to the active site of the enzyme than when these structures are present on the nontranscribed strand.

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