RNA polymerase II stalled at a thymine dimer: footprint and effect on excision repair

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ABSTRACT

Bulky lesions in the template strand block the progression of RNA polymerase II (RNAP II) and are repaired more rapidly than lesions in the non-transcribed strand, which do not block transcription. In order to better understand the basis of this transcription-coupled repair we developed an in vitro system with purified transcription and nucleotide excision repair proteins and a plasmid containing the adenovirus major late promoter and a thymine dimer in the template strand downstream of the transcription start site. The footprint of RNAP II stalled at the thymine dimer, obtained using DNase I, λ exonuclease and T4 polymerase 3'–5' exonuclease, covers ~40 nt and is nearly symmetrical around the dimer. The ternary complex formed at the lesion site is rather stable, with a half-life of ~20 h. Surprisingly, addition of human repair proteins results in repair of transcription-blocking dimers in the ternary complex. The blocked polymerase neither inhibits nor stimulates repair and repair is observed in the absence of CSB protein, the putative human transcription-repair coupling factor.

INTRODUCTION

DNA is template/substrate for enzyme systems which perform replication, transcription, repair and recombination. While the interactions of the individual enzyme systems with DNA are relatively well understood, the processing of DNA by more than one enzyme system has not been investigated in detail. It appears that when transcription and replication proceed in the same direction one can bypass the other with minimal interference; in contrast, when the replication fork opposes a transcribing RNA polymerase, replication pauses momentarily and then proceeds without disrupting the transcription complex (1–3) in both pro- and eukaryotic systems.

The joint actions of transcription and repair systems on damaged DNA lead to a phenomenon called transcription-coupled repair (4,5). Phenomenologically, transcription–repair coupling manifests itself by a faster rate of repair of transcribed DNA, in particular the template strand of transcribed DNA (5,6), compared with non-transcribed DNA. In Escherichia coli, the mechanistic basis of transcription–repair coupling is known: upon encountering a lesion in the template strand RNA polymerase (RNAP) stalls and forms a stable ternary complex; the DNA lesion within the complex is not readily accessible to the excision nuclease and as a consequence repair is inhibited (7). A transcription–repair coupling factor (TRCF) encoded by the mfd gene (8) releases the stalled RNA polymerase and recruits the damage recognition subunit of excision nuclease to the lesion site and thus accelerates the rate of damage recognition and hence repair (9).

In humans as well, transcription by RNAP II increases the rate of repair of the transcribed strand (6). Furthermore, humans appear to have a functional homolog of TRCF which is encoded by the CSB/ERCC6 gene (10,11). However, an in vitro system for transcription–repair coupling in humans is not available at present. Nevertheless, progress has been made towards developing such a system. In particular, it has been found that a cyclobutane thymine dimer (T<>T), which is subject to transcription-coupled repair (4), constitutes an absolute block for RNAP II (12) when present in the template strand but not when in the coding strand. In contrast, an acetylaminofluorene adduct, which does not undergo transcription-coupled repair (13), only causes brief pausing of RNAP II when located in the template strand (14). In the present study we have investigated the properties of the ternary complex of RNAP II at a T<>T in the template strand and the effect of the ternary complex on human excision nuclease. Our data show that RNAP II makes a stable ternary complex at the T<>T site in which RNAP II covers ~40 nt around the dimer in a nearly symmetrical manner. In contrast to the prokaryotic system, the stalled RNA polymerase, even in the absence of the presumptive coupling factor CSB, does not interfere with removal of the damage by the excision nuclease system.

MATERIALS AND METHODS

Template/substrates

pPU192, illustrated in Figure 1, possesses the adenovirus major late promoter (MLP) and a single thymine–thymine dimer located in the template strand at positions 149–150 downstream of the transcription start site. pPU192 also possesses a T7 RNAP promoter which is co-directional with the MLP. The dimer is located at nt 252–253 downstream of the T7 RNAP transcription start site. The DNA sequence in the region of the dimer is given...
Figure 1. Template/substrate pPU192. Start sites for transcription from the adenovirus major late promoter (MLP) and from a T7 RNAP promoter (T7) are indicated with arrows. The template possesses a single thymine cyclobutane dimer, indicated with the triangle, located in the template strand downstream from the promoters. Select restriction sites utilized for footprinting are shown.

in Figure 4A, pPU192 was labeled with $^{32}$P at either one of two locations indicated in Figure 4A and was constructed by previously described methods (15). pMLU112 contains the MLP and a downstream sequence ('U-less cassette') such that the first 112 nt of the transcript contains no U (16).

Transcription system

Transcription was reconstituted with purified recombinant (TBP, IIB, IIE and IIF) and native (IIH and RNAPII) human proteins (except yTBP) as described previously (17,18), with some modifications. Reactions were in transcription buffer (60 mM HEPES, pH 7.9, 6 mM Tris, pH 7.9, 108 mM KCl, 6.4 mM MgCl$_2$, 21.1 mM EDTA, 4 mM dithiothreitol, 2.8 mM $\beta$-mercaptoethanol, 5.5% glycerol and 3% polyethylene glycol). Template (~20 ng) was mixed with general transcription factors (GTFs) for 30 min at 28°C and then rNTPs were added to 625 $\mu$M each. To label with $[a-32P]$CTP were added. a-Amanitin when used was at 10 pg/ml. Incubation continued for 45 min. To analyze transcripts, reactions were extracted with phenol/chloroform, precipitated and RNA was resolved on sequencing gels. To footprint or repair transcribed template/substrate, transcription reactions were diluted with 2 vol. of a solution so as to arrive at repair buffer conditions (8.7 mM Tris, pH 7.9, 30 mM HEPES, pH 7.9, 61 mM KCl, 13 mM NaCl, 5.4 mM MgCl$_2$, 0.9 mM EDTA, 2 mM dithiothreitol, 0.9 mM $\beta$-mercaptoethanol, 5% glycerol, 1% polyethylene glycol, 1.9 mM ATP, 0.21 mM each GTP, CTP and UTP, 20 $\mu$M each dNTP, 133 $\mu$g/ml BSA and 17 $\mu$g/ml pMC1). pMC1 was added as competitor DNA for non-specific DNA binding proteins. At this point reagents for footprinting and/or repair were included and further processing of reactions was as described below.

Transcription by T7 RNAP was with 12 U enzyme (Promega) directly in repair buffer.

Footprinting

DNase I (Gibco BRL) digestion was with several hundred units of enzyme for 5 min in the presence of 2 $\mu$M CaCl$_2$. Products were extracted with phenol, precipitated and resolved on 8 or 10% sequencing gels. Digestion with $\lambda$ exonuclease (Pharmacia) was with 27 U enzyme in repair buffer after cleaving the plasmid with PvuII and HaeIII. Digestion with 2 U T4 polymerase (BMB) was after digesting the plasmid with HaeIII and then diluting the 30 $\mu$l reaction to 100 $\mu$l with 1x commercial T4 polymerase reaction buffer.

Repair systems

Preparation of cell-free extract (CFE) was as described previously (19). Excision assay with CFE was under the conditions described above for 25 min at 29°C. After repair, extraction with phenol and precipitation in ethanol, the DNA was resolved on an 8 or 10% sequencing gel to identify the radiolabeled 24–29 nt products of the nucleotide excision reaction.

Excision assay with a reconstituted system of partially purified native human general repair factors (GRFs) was also used. GRFs were generated based upon a reproducible purification scheme previously described (20), with the exception that we used native human XPA rather than recombinant protein from E.coli. All of the repair proteins passed through the first column, DE-52. We found by complementation analysis that XPA passed through the second column, affigel blue, and highly active material was obtained following two additional passes through the affigel blue column. The ERCC1–XPF complex, which is eluted from the affigel blue column with low salt, was further purified on a heparin column and a second preparation of ERCC1–XPF was additionally purified on an MBP-XPA affinity column. XPC and TFIIH, which partially co-elute from the affigel blue column at intermediate salt, were further purified. XPG, which co-elute when the affigel blue column is washed with high salt, was also separated from one another on an SP-Sepharose column. In some experiments we used recombinant XPG purified from insect cells (21). In our system, omission of any repair protein individually, except XPG, abolished repair. XPG is known to partially co-purify with TFIIH by this procedure (20). The optimal amount of each GRF used for repair was determined empirically. This reconstituted repair system was found to be several times more active than CFE. An activity (described in Results) which removes RNAP II stalled at a T<>T was largely removed from the reconstituted system. The RNAP releasing activity co-purified with TFIIH and XPC through the DE-52 and affigel blue columns but it did not bind to the third column, SP-Sepharose, which retained both XPC and TFIIH.

To assay repair of lesions where RNAP II was stalled, transcription reactions were first photoreactivated with 5 nM E.coli DNA photolyase. This procedure was used to remove T<>T located on templates where RNAP II was not stalled. In controls conducted in the absence of photoreactivating light, this addition of photolyase was found to inhibit nucleotide excision repair by <20%. After photoreactivation, reactions were diluted into repair buffer with GRFs and incubated at 29°C for 25 min in the absence of photoreactivating light. In parallel reactions, DNase I footprinting was performed during an additional 5 min incubation (after the 25 min reaction) to determine the percentage of templates having a stalled polymerase. In additional parallel reactions, after photoreactivation, a low level of pPU192 (3–12% of the original 20 ng added) was added to samples that had undergone both mock transcription in the presence of a-amanitin and then photoreactivation. After excision repair, reactions were then digested with proteinase K, extracted with phenol, precipitated with ethanol and resolved on an 8% sequencing gel. In processing DNase I digestion products, the proteinase K digestion was omitted. Products were quantified with a phosphorimager.
With the reconstituted transcription system we first confirmed the formation and stability of a ternary complex at a T<>T before it stops (Fig. 1), as previously reported (12). A low level of transcript (data not shown). The truncated RNA was -145-150 nt in length, compared with transcripts made from a control undamaged plasmid. The presence of the dimer resulted in the formation of a truncated complex formed at a T<>T site. Figure 2 shows the results of an experiment conducted at room temperature over a 8 day period.

RESULTS

Formation and stability of a ternary complex at a T<>T

With the reconstituted transcription system we first confirmed the observation that a T<>T in the template strand blocks progression of RNAP II (12). Radiolabeled transcripts made from pPU192 were compared with transcripts made from a control undamaged plasmid. The presence of the dimer resulted in the formation of a truncated transcript (data not shown). The truncated RNA was -145-150 nt in length, indicating that RNAP II transcribes very close to the lesion before it stops (Fig. 1), as previously reported (12). A low level of transcription past the dimer site (~1%) was observed, a result of either transcriptional bypass of the dimer or trace contamination of the damaged pPU192 with undamaged plasmid.

To examine the status of RNAP II upon encountering a T<>T we conducted footprinting experiments. To obtain the footprint the radiolabel was incorporated at the 13th phosphodiester bond downstream of the T<>T in pPU192 and following incubation with GTFs and RNAP II, the DNA was digested exhaustively with DNase I and then analyzed on sequencing gels. Using this procedure 30-48 nt fragments of the transcribed strand were protected from DNase I (data not shown). The truncated RNA was -145-150 nt in length, indicating that RNAP II transcribes very close to the lesion before it stops (Fig. 1), as previously reported (12). A low level of transcription past the dimer site (~1%) was observed, a result of either transcriptional bypass of the dimer or trace contamination of the damaged pPU192 with undamaged plasmid.

Footprint of RNAP II stalled at a T<>T

To understand the interactions of the transcription apparatus with the nucleotide excision repair system it is useful to know the positioning of the stalled RNAP II around the dimer. Therefore, the boundaries of the DNase I footprint of stalled RNAP II were determined. Footprints such as in Figure 2 were gel purified and then digested with either PvuII and HaeIII or T4 polymerase (after digestion with HaeIII). Arrows indicate where the exonucleases stop upon encountering the blocked RNAP II. PvuII cleaves 66 nt upstream of the T<>T and the product of λ exonuclease digestion is 94 nt in length. HaeIII cleaves 46 nt downstream and the products of T4 polymerase digestion are 66, 67 and 71 nt in length. Due to the many non-specific DNA binding proteins among the GTFs, a number of non-specific exonuclease stop sites are observed when GTFs are present, as in lanes 3 and 4. The origin of the band 104 nt in size in lane 8 is unclear. It may result from a population of RNAP II that arrests at a sequence-dependent pause site well before reaching the lesion or from polymerases that stop when they encounter an RNAP II already at the dimer ahead. DNA ladders were run in lanes L and DNA markers of the sizes indicated were run in lanes M.

Footprint of ternary complex stalled at a lesion. Using the pPU192 template, the borders of RNAP II blocked at the dimer were directly probed with λ exonuclease (after digestion with PvuII and HaeIII) or T4 polymerase (after digestion with HaeIII). Arrows indicate where the exonucleases stop upon encountering the blocked RNAP II. PvuII cleaves 66 nt upstream of the T<>T and the product of λ exonuclease digestion is 94 nt in length. HaeIII cleaves 46 nt downstream and the products of T4 polymerase digestion are 66, 67 and 71 nt in length. Due to the many non-specific DNA binding proteins among the GTFs, a number of non-specific exonuclease stop sites are observed when GTFs are present, as in lanes 3 and 4. The origin of the band 104 nt in size in lane 8 is unclear. It may result from a population of RNAP II that arrests at a sequence-dependent pause site well before reaching the lesion or from polymerases that stop when they encounter an RNAP II already at the dimer ahead. DNA ladders were run in lanes L and DNA markers of the sizes indicated were run in lanes M.

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To examine repair where RNAP II was stalled we performed the transcription-repair coupling in extracts. The transcription system was used to form stalled ternary complexes as outlined in Figure 6. First, a reconstituted RNAP II stalled at a dimer does not inhibit excision and functional assays show that CFEs contain an activity which prevents elongation upon addition of UTP. Thus, both physical and formation of 5' and 3' incisions. Hence these findings raise the possibility that a stalled complex might interfere with the assembly of excision nuclease and formation of 5' and 3' incisions.

Cell-free extract removes stalled RNAP II

To examine repair of transcription-blocking lesions, repair-competent extracts were added to reactions in which RNAP II was stalled at a dimer. Results could not be interpreted because, unexpectedly, the extract, whether prepared from wild-type HeLa or CSA- or CSB- mutant cells, released RNAP II from the template, as determined by footprinting experiments (data not shown). An RNAP II 'release' factor, or 'factor R', was partially purified and its activity is shown in Figure 5. In this assay of factor R, RNAP II was stalled by nucleotide starvation at the end of a 112 nt long U-less cassette and then incubated with and without factor R. UTP was then added and transcription proceeded to the PvuII site where the template had been cleaved. Factor R prevented elongation upon addition of UTP. Thus, both physical and functional assays show that CFEs contain an activity which disrupts the ternary complex making it impossible to study transcription-repair coupling in extracts.

RNAP II stalled at a dimer does not inhibit excision

To examine repair where RNAP II was stalled we performed the experiments outlined in Figure 6. First, a reconstituted transcription system was used to form stalled ternary complexes at the dimer in pPU192. This system transcribes 3–10% of input plasmids. The remaining plasmids were photoreactivated by treatment with E.coli DNA photolyase plus near-UV light. This treatment does not repair dimers where RNAP II is stalled (12) and thus results in two major populations of DNA: (i) plasmid with no dimer and no RNAP II; (ii) plasmid with RNAP II stalled at a dimer. At this point, instead of performing repair with CFE, which removes polymerase, or highly purified GRFs, which do not efficiently repair circular substrates (data not shown), we used partially purified human GRFs.

Before examining repair, we tested whether the partially purified repair factors removed stalled RNAP II from the dimer. In Figure 7A, the footprint of RNAP II blocked at the dimer in pPU192 (lane 2) was examined after incubation with and without GRFs. Although there is a high background of DNase I protection caused by the DNA binding of RPA (lanes 5 and 6) and the 24–29 nt excision product seen in lane 7 partially overlaps the footprint in lane 8, it is evident from both a visual inspection of the autoradiograph and from quantitative values obtained by phosphorimaging of the gel that the repair factors did not remove the stalled polymerase from the site of the dimer. We next examined repair where polymerase was blocked using the procedure outlined in Figure 6. Results are shown in Figure 7B. Reaction conditions were the same as those used in Figure 7A, except DNase I was omitted. In each repeat of this experiment we measured the percentage of templates transcribed. In this particular experiment, lanes 1 and 2 (Fig. 7B) show the footprint of the stalled polymerase in the absence of GRFs. Comparison of the amount of protected DNA in lane 2 with the amount of intact DNA at the top of the gel in lane 3 (not shown) reveals that in this experiment 3% of the templates had RNAP II blocked at the dimer. Lanes 3–8 show excision reaction products, which are 25–28 nt in size. Lanes 1 and 2 and 3–8 are from the same experiment. Thus, in the excision assay where RNAP II was stalled at the dimer and then the free dimers were photoreactivated, the amount of signal in lane 2 reflects the amount of substrate dimer in ternary complex and potentially available for repair.
Figure 6. Assay to detect repair of dimers within a blocked RNAP II complex. The diagram illustrates a procedure to detect repair of lesions where RNAP II is stalled. The objects with small triangles represent plasmids with a dimer. Transcription (in the absence of α-amanitin) by a reconstituted transcription system results in the formation of RNAP II stalled at the dimer (represented by a small circle with a RNA tail) on a few percent of the input templates. Photoreactivation removes the dimers from templates not engaged in transcription. Repair-competent CFE or partially purified GRFs are added to test for repair of the dimer where RNAP II is stalled.

Lane 5 shows the amount of repair actually observed after subsequent incubation with repair factors. As a control, lane 4 contained DNA that was photoreactivated in the absence of transcription. The faint signal in lane 4 reflects repair of a small amount of non-photoreactivated dimers and possibly a trace of template with non-dimer lesions. Most importantly, the greater level of repair in lane 5 (+ transcription) compared with lane 4 (-transcription) indicates the amount of repair of dimers where RNAP II was stalled.

Next we asked whether the presence of RNAP II stalled at the dimer stimulated or inhibited repair. To address this, we performed a ‘standard addition’ reaction, which is shown in lane 6 of Figure 7B. In this reaction there was no transcription but there was photoreactivation, exactly as in lane 4. However, before adding repair factors as in lane 4, we added fresh, non-photoreactivated substrate, the amount of which was 6% of the original input DNA. The repair signal from this DNA (lane 6) was ~2-fold the signal from substrate in ternary complex (lane 5), which contained 3% of the original input substrate (lane 2). Thus, the polymerase did not strongly stimulate or inhibit repair of the transcription-blocking dimer. In three separate experiments, the percentage of repair of transcription-blocking dimers relative to repair in standard addition reactions containing a comparable amount of input substrate was 85, 72 and 112%.

Control experiments showed that the transcription signal increased linearly with added substrate when 3–12% of the original amount of input substrate was tested. Excision repair after photoreactivation was done under yellow light, to prevent photoreactivation in standard addition reactions. Furthermore, lanes 7 and 8 tested whether the amount of photolyase used would inhibit repair in standard addition reactions. As can be seen, photolyase did not substantially inhibit human excision repair, as very high concentrations are known to do (23–24). Because of a lack of antibodies we do not know if the reconstituted systems contain CSA protein. However, using anti-CSB antibodies we have found only a trace amount of CSB protein. Thus, we conclude that even in the absence of CSB, RNAP II stalled at a dimer does not inhibit or stimulate human excision nuclease, even though it completely covers the 3' excision site.
For comparison with the human transcription system, we tested RNAP II stalled at a dimer inhibits repair template strand but is not blocked by adducts in the complementary strand (12). This blockage of RNAP II is indicated with arrows. A 24 nt DNA size marker in lane M is indicated.

**Figure 8.** Inhibition of human excision nuclease by T7 RNAP stalled at a T<>T. pPU192 was incubated with and without T7 RNAP under the conditions indicated and then HeLa CFE was added. The 24–28 nt products of excision are indicated with arrows. A 24 nt DNA size marker in lane M is indicated.

**T7 RNAP stalled at a dimer inhibits repair**

For comparison with the human transcription system, we tested the effect of a T7 RNAP blocked at the dimer on the human repair enzyme. Transcription of pPU192 from the T7 RNAP promoter was controlled by adding or withholding polymerase and rNTPs. In contrast to the human polymerase, T7 RNAP is highly efficient and transcribes nearly 100% of templates. Therefore, we simply incubated pPU192 briefly with T7 RNAP and rNTPs to form stalled elongation complexes, then repaired the DNA with human CFE. The results in Figure 8 show that, in contrast to human RNAP II, T7 RNAP II stalled at the dimer prevents repair of the dimer by human excision nuclease.

**DISCUSSION**

The progress of polymerases, including human RNAP II, has been shown to be impeded by bulky adducts located in the template strand but is not blocked by adducts in the complementary strand (12). This blockage of RNAP II is probably an early event in the human transcription-stimulated repair reaction (14), as it is in *E.coli*. Footprints of human RNAP II stalled by nucleotide starvation have been described. Ternary complexes stalled at different sites demonstrated DNase I footprint sizes that ranged from 42 to 58 nt. Interestingly, more of the coding strand was protected than the template strand and complexes stalled at different sites on the template exhibited slightly different footprint sizes (25–27). Exonuclease III footprints of ternary complexes generated by nucleotide starvation were 33–39 bp in size (28). Regions of protection obtained with the different footprinting procedures were fairly symmetrical around the 3'-end of the RNA and stalled elongation complexes were reported to be stable. Thus, the footprint and stability of RNAP II blocked at a dimer are generally similar to those of RNAP II stalled by nucleotide starvation and the structural feature that elicits preferential repair may be not more than the combination of a stationary polymerase in a ternary complex and the bulky DNA adduct.

By using purified transcription and repair proteins we were able to examine repair in the absence of removal of RNAP II. With this system and using enzymatically purified ternary complexes we observed repair of the dimer within the complex. The presence of the polymerase had no detectable inhibitory or stimulatory effect on repair. This result was unexpected for several reasons. First, in a comparable system with purified *E.coli* transcription and repair proteins, the stalled polymerase did inhibit repair (7). Also, stalled human RNAP II was found to partially or completely ‘cover’ the incision sites of human excision nuclease. Furthermore, in the ternary complex, the 3’ incision site and lesion may be in a region or adjacent to a region of single-stranded DNA or a DNA–RNA hybrid, as shown in Figure 4. Finally, RNAP II blocked at a T<>T is known to prevent repair of the T<>T by *E.coli* DNA photolyase (12). Our results do not have the resolution to answer whether or not the ternary complex is removed or remains bound after the dual incision.

Our finding that repair occurs in a ternary complex containing a transcription bubble terminating at a photodimer suggests that a dimer adjacent to a synthetic transcription bubble should be an efficient substrate for human excision nuclease. This prediction has recently been confirmed in a model system in which the T<>T was preceded on the 3’-side by a bubble of 10 mismatched nucleotides (Mu and Sancar, unpublished results). The effect of an RNA–DNA hybrid on repair has not yet been tested.

In *E.coli*, RNAP stalled at a dimer inhibits nucleotide excision repair of the dimer in vitro (7). The *E.coli* Mfd protein couples repair to transcription by removing the polymerase stalled at lesions in the template strand and delivering the repair enzymes to the lesion. In UV-irradiated *E.coli* cells which are mfd<sup>–</sup> and lack the transcription–repair coupling factor, specific inhibition of repair by stalled RNAP results in an elevated frequency of mutation, specifically in the template strand (29). Since human RNAP II stalled at a dimer does not inhibit repair, it is predicted that the strand bias for mutation in mfd<sup>–</sup> cells is absent from CSA<sup>+</sup> and CSB<sup>+</sup> human cells, which lack the presumed human coupling factors.

A relevant observation has been made with Rad 26 disruption mutants of *Saccharomyces cerevisiae*. Rad 26, which is homologous to human CSB, is the yeast transcription–repair coupling factor (30). In certain genetic backgrounds, transcription-stimulated repair was observed even in the absence of Rad 26 protein (31). This finding is consistent with transcription–repair coupling resulting from a combination of coupling factor-independent and -dependent pathways. It is possible that the coupling factor-independent pathway involves a release from inhibition. Chromatin structure inhibits repair (32,33). Consequently, when a lesion in the template strand blocks RNAP II it may become more accessible to repair enzymes than when constrained within a nucleosome.

Recent investigations of coupling factor-dependent repair have shown that purified CSB protein does not remove a polymerase stalled at a dimer (18) as does its *E.coli* counterpart (Mfd) (9). However, CSB does bind to human RNAP II (Selby and Sancar, unpublished result) and to human GRFs (18,34) and thus may enhance the repair rate by recruiting repair enzymes to the transcription-blocking lesion, as is the case in *E.coli* (9).

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REFERENCES