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TFIIH-mediated nucleotide excision repair and initiation of mRNA transcription in an optimized cell-free DNA repair and RNA transcription assay

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ABSTRACT

In mammalian cells, mRNA transcription is initiated with the aid of transcription initiation factors. Of these, TFIIH has also been shown to play an essential role in nucleotide excision repair (NER), which is a versatile biochemical pathway that corrects a broad range of DNA damage. Since the dual role of TFIIH is conserved among eukaryotes, including yeast and mammalian cells, the sharing of TFIIH between NER and RNA transcription initiation might provide some survival advantage. However, the functional relationship between NER and RNA transcription initiation through TFIIH is not yet understood. We have developed an optimized cell-free assay which allows us to analyze NER and RNA transcription under identical conditions. In this assay, NER did not compete with RNA transcription, probably because the extracts contained sufficient amounts of TFIIH to support both processes. Thus, NER can be considered functionally independent of RNA transcription initiation despite the fact that both processes use the same factor.

INTRODUCTION

The basal transcription factor TFIIH has a dual role in eukaryotes, being involved in both initiation of mRNA synthesis by RNA polymerase II (Pol II) and in nucleotide excision repair (NER) (1–4). This factor from human cells contains multiple subunits, including XPB (2), XPD (5) and p62 (6). XPB and XPD are 3′→5′ (7) and 5′→3′ (8) helicases respectively. In studies with yeast mutants of TFIIH, the 3′→5′ helicase has been shown to be essential for both NER and initiation of mRNA synthesis (9), while the 5′→3′ helicase is required only for NER (10,11).

Along with TFIIH, initiation of mRNA synthesis requires the basal transcription factors TFIIB, TFIID, TFIIE, TFIIF and TFIIJ. These factors form a preinitiation complex at the TATA box and Pol II is loaded onto DNA during the process of complex formation (12). To facilitate RNA synthesis, the template DNA is probably melted by the TFIIH-associated helicase activities, resulting in activation of Pol II (2). In living organisms, a certain amount of these factors must be produced to maintain competence in transcription, since mRNA synthesis is essential for growth and viability of cells (9,13).

NER is a multienzymatic process that, in human cells, is initiated by binding of the XPA–RPA complex to DNA damage (14–17). Then, an oligonucleotide of 27–30 bases containing the damage is excised from the DNA (18) by DNA nucleases, the XPF–ERCC1 complex (19) and the XPG protein (20). The helicase activities of TFIIH are also required for excision (1). The gapped duplex resulting from excision is filled by repair synthesis, utilizing RPA (21), PCNA (22), DNA polymerases (23) and DNA ligase (24), which are also needed for DNA replication (25). This repair pathway removes a broad spectrum of DNA damage, including UV-induced cyclobutane pyrimidine dimers and 6–4 pyrimidine-pyrimidone photoproducts (26). In humans, NER deficiency results in the hereditary disorder xeroderma pigmentosum (XP), characterized by hypersensitivity to sunlight exposure and greatly increased skin tumor formation (27).

The dual functions of TFIIH are conserved among eukaryotes, including yeast (4) and mammals (1,3). Thus, eukaryotic cells may acquire a certain survival advantage by using TFIIH in both mRNA transcription initiation and NER. However, the functional relationship between NER and RNA transcription initiation through TFIIH is not yet understood.

Therefore, we have developed an optimized cell-free DNA repair–RNA transcription system which allowed us to analyze NER and RNA transcription under identical assay conditions and we have investigated the relationship between NER and transcription initiation through TFIIH. Since NER did not compete with RNA transcription in our assay system, we conclude that NER is functionally independent of RNA transcription initiation.

MATERIALS AND METHODS

Materials

RNase T1 was purchased from Gibco BRL. [α-^{32}P]NTPs and [α-^{32}P]dNTPs were obtained from Amersham. *Escherichia coli* endonuclease III and mouse antibody against RPA 70 kDa subunit (70C; 21) were kindly provided by Dr R.D.Wood. Purified TFIIH from HeLa cells (2,28), mouse monoclonal antibody against the p62 subunit of TFIIH (3C9; 29) and antibody to TFIIE (2A1) were kindly provided by Dr J.-M.Egly.

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Lymphoblastoid cells, GM01953C (normal) and GM01857 (Cockayne's syndrome group A; CSA), were obtained from the NIGMS Human Mutant Cell Repository, NJ, and cultured in RPMI 1640 supplemented with 20% fetal bovine serum and antibiotics. HeLa S3 cells were maintained in suspension culture with MEM supplemented with 5% fetal bovine serum and antibiotics.

Preparation of cell-free extracts

Actively growing cells were collected by centrifugation and dead cells, which were often found even in optimized culture conditions, were removed with Ficoll-Paque (Pharmacia) as described (30). The cell-free extracts were prepared by the method of Manley *et al.* (31). In some experiments, TFIIH-depleted extracts were employed. To prepare the extracts, antibody against the p62 subunit of TFIIH (3C9) was incubated with extract for 60 min at 0°C and the antibody–TFIIH complex was removed with protein G–Sepharose (GammaBind Plus Sepharose, Pharmacia Biothec). About 95% of the TFIIH was removed from the extract (designated 95% TFIIH-depleted extract) by this procedure, as determined by Western blotting, using antibody against the p62 subunit for the analysis.

Construction of a template plasmid for RNA transcription by Pol II

pGf1 (Fig. 1A) contained the adenovirus major late promoter (AdMLP) and a G-less cassette (32), which does not have any guanine residues in the non-transcribed strand. It was constructed from pAdHap (3.1 kb) (33). To replace an *Ssp*I site in pAdHap with a *Pst*I site (pAdHap-s), the plasmid was digested with *Ssp*I, ligated to a *Pst*I linker and the linker digested with *Pst*I. The plasmid was purified by 1% agarose gel electrophoresis containing 1 μg/ml ethidium bromide (EtBr) and transfected into *E.coli* after ligation of the *Pst*I site. A *Mae*I site (labeled M in Fig. 1A) was then replaced with a *Pac*I site (pAdHap-ps). Since there were three other *Mae*I sites (labeled m in Fig 1A), pAdHap-s was digested with *Pst*I and *Bam*HI and the large fragment (2.2 kb) and the small fragment (0.8 kb) containing the *Mae*I site (labeled M in Fig. 1A) were purified by 1% agarose gel electrophoresis containing EtBr. The small fragment was then digested with *Mae*I and the *Pst*I–*Mae*I fragment (0.6 kb) was purified by 2% agarose gel electrophoresis containing EtBr. This *Pst*I–*Mae*I fragment was ligated with the large fragment (2.2 kb) at the *Pst*I site and the resulting *Bam*HI–*Mae*I fragments were purified by 1% agarose gel electrophoresis containing EtBr. This fragment was then treated with mung bean nuclease, ligated with a *Pac*I linker, digested with *Pac*I, purified by 1% agarose gel electrophoresis containing EtBr and transfected into *E.coli* after ligation of the *Pac*I site. The resulting pAdHap-ps plasmid was digested with *Pac*I and *Hin*dIII and the 2.7 kbp fragment was purified for ligation with the G-less cassette.

To construct the G-less cassette, a synthetic 60 base G-less oligonucleotide was annealed with a synthetic 60 base C-less oligonucleotide containing a 10 base sequence complementary to the 3′ site of the G-less oligonucleotide (Fig. 1A). Then, double-stranded DNA (110 bp) was synthesized by incubation of the annealed oligonucleotide with Klenow DNA polymerase. The resulting DNA fragment contained *Pac*I and *Hin*dIII sites at either end. This fragment was then digested with *Hin*dIII and *Pac*I, ligated with the 2.7 kb fragment from pAdHap-ps and transfected into *E.coli*. The resulting plasmid contained AdMLP and the G-less cassette (pGf1, 2.8 kb). The sequence of the non-transcribed strand of this G-less cassette is as follows: 5′-TCCTCACTCTCTTCCTCTTAATTAACCCCTACCCTCC-CACCACACCTCTTAACTAATATTAACTCACTCCCCTACC-CCATACACCACTCTACCTCACCCCATCAACCTTAA-3′.

pΔGf1 was prepared from plasmid pGf1 by removing AdMLP. To excise the 80 bp fragment containing AdMLP from pGf1, the plasmid was digested with *Pac*I and *Eco*RI. Then, a synthetic 50 bp oligonucleotide containing *Pac*I and *Eco*RI sites at either end was ligated to the 2.8 kb fragment of pGf1 to construct pΔGf1.

Cell-free DNA repair, RNA transcription and DNA repair–RNA transcription assay

The typical reactions were carried out with cell-free extracts, 0.75 μg pGf1 containing 430 fmol AdMLP and 0.25 μg UV-irradiated pBluescript II KS$^+$ (pBS, 3.0 kb; Stratagene), which was prepared by irradiation with UV (450 J/m^2 at 254 nm), followed by treatment with *E.coli* endonuclease III as described by Wood *et al.* (34). To make the amount of pBS commensurate with that of pGf1, 0.5 μg non-damaged pBS was also added to the reaction. Based upon the report of Jones *et al.* (35), the level of cyclobutane pyrimidine dimers and 6–4 pyrimidine-pyrimidone photoproducts in 0.25 μg UV-irradiated pBS was estimated to be 1300 and 430 fmol respectively. It has been shown that the 6–4 pyrimidine-pyrimidone photoproduct is ~10 times better as a substrate than the cyclobutane pyrimidine dimer for NER in cell-free assays (36). In some reactions, pGf1 was replaced with pΔGf1 and UV-irradiated pBS was replaced with non-damaged pBS.

The reactions were carried out in one of four different reaction mixtures.

Repair assay. The reaction mixture was that used in the cell-free DNA repair assay reported by Wood *et al.* (34) and contained 20 mM HEPES–KOH, pH 7.9, 2 mM ATP, 8 μM dATP, 25 μM each of dCTP, dTTP and dGTP, 40 mM phosphocreatine, 13 U/ml creatine phosphokinase (CPK), 70 mM KCl, 5 mM $MgCl_2$, 3.4% glycerol, 300 μM dithiothreitol, 300 μg/ml bovine serum albumin (BSA) and 0.75 μCi [α-^{32}P]dATP (1 μCi/150 pmol).

Transcription assay. This was the slightly modified reaction mixture used in cell-free RNA transcription assays reported by Dignam *et al.* (37) and contained 20 mM HEPES–KOH, pH 7.9, 600 μM each of ATP, UTP and GTP, 25 μM CTP, 40 mM phosphocreatine, 13 U/ml CPK, 0.3 U RNase T1, 60 mM KCl, 10 mM $MgCl_2$, 3.4% glycerol, 300 μM dithiothreitol, 300 μg/ml BSA and 2.1 μCi [α-^{32}P]CTP (1 μCi/300 pmol).

Repair–transcription assay (unoptimized). The reaction mixture for a DNA repair–RNA transcription assay was the same as for the transcription assay, but the assay also contained dNTPs at the same concentrations as in the repair assay and the reactions were carried out with either 0.75 μCi [α-^{32}P]dATP (1 μCi/150 pmol) for repair or 2.1 μCi [α-^{32}P]CTP (1 μCi/300 pmol) for RNA transcription.

Repair–transcription assay (optimized). The reaction mixture for an optimized DNA repair–RNA transcription assay contained 20 mM HEPES–KOH, pH 7.9, 1.2 mM ATP, 360 μM GTP, 180 μM UTP, 270 μM CTP, 600 μM dATP, 180 μM dGTP, 120 μM dTTP, 480 μM dCTP, 10 mM phosphocreatine, 40 U/ml CPK, 0.3 U RNase T1, 60 mM KCl, 10 mM $MgCl_2$, 3.4%

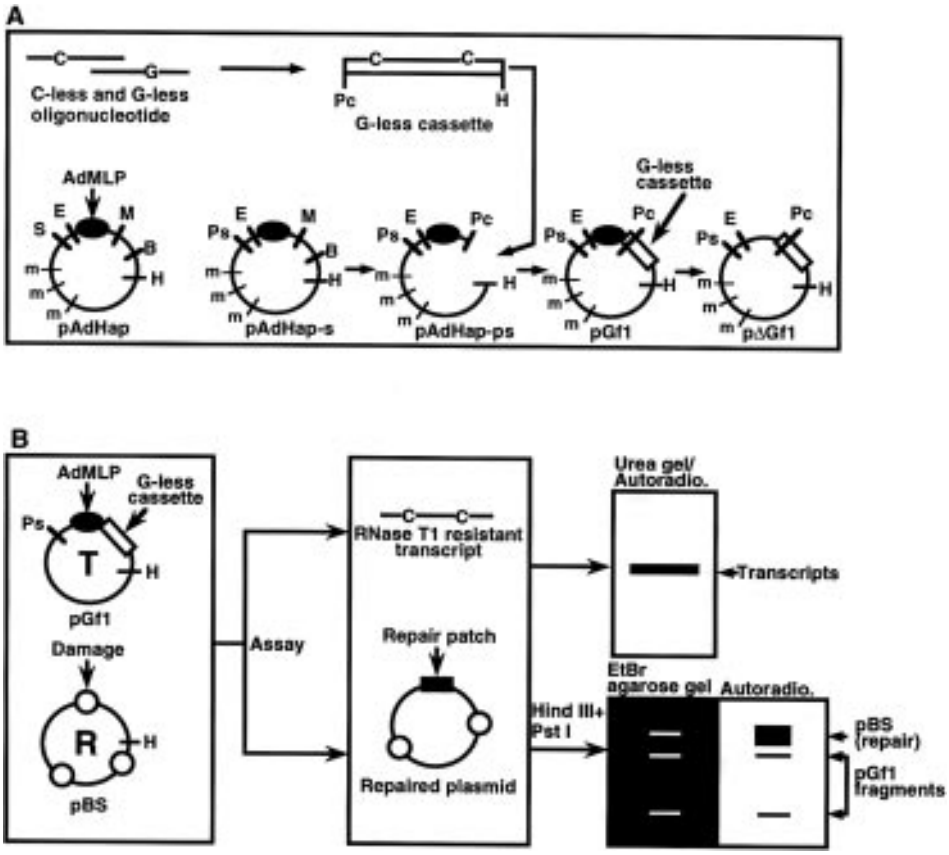

Figure 1. Schematic outline for the construction of pGf1 and pΔGf1 (**A**) and the DNA repair–RNA transcription assay (**B**). pGf1 was constructed from pAdHap (33) and pΔGf1 from pGf1 (see Materials and Methods for details). S, *Ssp*I site; H, *Hind*III site; Ps, *Pst*I site; Pc, *Pac*I site; E, *Eco*RI site; B, *Bam*HI site; M and m, *Mae*I sites; C, cytosine residue; G, guanine residue. Details for the DNA repair–RNA transcription assay are described in Results. T, plasmid DNA for RNA transcription by Pol II; R, plasmid DNA for NER.

glycerol, 300 µM dithiothreitol, 300 µg/ml BSA and either 30 µCi [α-^{32}P]dGTP (1 µCi/150 pmol) for repair or 30 µCi [α-^{32}P]UTP (1 µCi/150 pmol) for transcription. In the repair–transcription assay (optimized), the specific activity of [α-^{32}P]UTP was twice that of [α-^{32}P]CTP in the transcription assay and repair–transcription assay (unoptimized), since the number of thymidine residues in the transcripts from the G-less cassette was half the number of cytosine residues.

These cell-free reactions were carried out in 25 µl volumes at 30°C for 60 min and were terminated by addition of 25 µl 1.2% SDS, 40 mM EDTA and 2.4 mg/ml proteinase K followed by incubation for 30 min at 37°C. Plasmid DNA and RNase T1-resistant transcripts were purified by phenol/chloroform extraction and precipitated with ethanol with ammonium acetate and tRNA. For analysis of DNA repair, plasmid DNA was incubated with 50 U *Hin*dIII, 50 U *Pst*I and 75 µg RNase A in a 60 µl reaction mixture for 30 min at 37°C and 20 µl was fractionated by electrophoresis on a 1% agarose gel containing 1 µg/ml EtBr. A photographic negative of the gel was taken to analyze DNA recovery and phosphorimaging (GS-363 Molecular Image System; BioRad) and autoradiography of the dried agarose gel were used to determine repair activity. For RNA transcription, ethanol-precipitated RNase T1-resistant transcripts were dissolved in gel loading buffer containing 7 M urea, 10 mM EDTA, 5% glycerol, 0.05% bromophenol blue and 0.05% xylenene cyanol, denatured at 65°C for 10 min and fractionated on a 10% acrylamide–4 M urea gel. Phosphorimaging and autoradiography of the dried urea gel were used to quantify the RNA.

Analysis of dNTP and NTP degradation

The cell-free reactions were carried out with pGf1, UV-irradiated pBS and non-damaged pBS as described above in the presence of either 0.5 µCi [α-^{32}P]dATP, [α-^{32}P]dGTP, [α-^{32}P]dCTP, [α-^{32}P]dTTP, [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP or [α-^{32}P]UTP. After incubation for 60 min at 30°C, the reactions were terminated by addition of 25 µl loading buffer. Samples were fractionated by electrophoresis on 10% polyacrylamide–4 M urea gels. Phosphorimaging and autoradiography of dried gels were used to visualize the radioactivity.

Western blotting

Cell-free extracts (25 µg) were denatured in gel loading buffer for 5 min at 100°C, fractionated on an SDS–12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was then incubated with mouse antibody against the p62 subunit of TFIIH in buffer containing 0.1% Tween-100, 150 mM NaCl and 10 mM Tris–HCl, pH 7.5, (TBST) with 5% skimmed milk overnight at 4°C. After washing the membrane four times for 5

min with TBST, it was incubated with anti-mouse secondary antibody conjugated with horse radish peroxidase (HPR-Goat anti-mouse IgG; Zymed Laboratories Inc., San Francisco, CA) in TBST containing 5% skimmed milk for 1 h at 25°C. The membrane was then washed four times for 5 min with TBST and incubated with chemiluminescence reagents (Renaissance; Du Pont). Protein was visualized by exposure of the membrane to X-ray film. Over six different exposure times were taken and the films were scanned (Scan Jet IIP, Hewlett Packard) for quantitation.

RESULTS

Outline for DNA repair–RNA transcription assay

To establish a DNA repair–RNA transcription assay which allows analysis of NER and transcription through TFIIH under identical assay conditions, the cell-free assay for NER and RNA transcription was carried out with two different kinds of closed circular plasmid DNA (Fig. 1B): pGf1 for RNA synthesis by Pol II and UV-irradiated pBS for NER.

pGf1 contained AdMLP and a G-less cassette (32), which is a stretch of DNA without guanine residues in the non-transcribed strand. Thus the transcripts did not contain guanine residues and they were resistant to RNase T1 in the reaction mixture. After fractionation by urea gel electrophoresis, the RNA transcription activity of Pol II was determined by quantitation of ^{32}P in the transcripts.

UV-irradiated pBS contained pyrimidine dimers and 6–4 pyrimidine-pyrimidone photoproducts. After digestion of plasmid DNA with *Pst*I and *Hin*dIII and separation of UV-irradiated pBS from fragments of pGf1 by EtBr–agarose gel electrophoresis, repair activity was determined by comparing the amount of ^{32}P in UV-irradiated pBS with that in fragments of pGf1.

Optimization of reaction conditions

The originally reported DNA repair assay found the optimal KCl concentration to be between 40 and 100 mM and was carried out at pH 7.9 with 2 mM ATP, 8 μM dATP, 25 μM each of dCTP, dTTP, dGTP and an ATP-regenerating system with 13 U/ml CPK (34). For transcription, the optimal KCl concentration and pH reported by Dignam *et al*. (37) are 60 mM and between 7.5 and 8.5 respectively and the reaction contained 600 μM each of ATP, UTP and CTP and 25 μM GTP. To establish a DNA repair–RNA transcription assay, we have tested an assay with 60 mM KCl, HEPES–KOH, pH 7.9, 8 μM dATP, 25 μM each of dCTP, dTTP and dGTP, 2 mM ATP, 600 μM each of UTP and GTP, 25 μM CTP and an ATP-regenerating system with 13 U/ml CPK (see Materials and Methods for detailed reaction conditions).

However, in this assay [designated repair–transcription assay (unoptimized)] NER activity was significantly reduced compared with that in the assay carried out under the previously reported conditions (designated repair assay; see Materials and Methods) (Fig. 2A, lanes 1 and 3). Such a reduction may be due to increased degradation of dCTP. As shown in Figure 3 (dCTP, lane 3), the recovered dCTP after incubation with 100 μg cell-free extract for 60 min incubation in this assay was significantly reduced and a degradation product, which migrated more slowly than dCTP on the urea gel, had appeared. However, reactions with a higher initial dCTP concentration (480 μM) did not exhibit this reduction of dCTP (Fig. 3, dCTP, lane 4) and resulted in increased NER activity (Fig. 2A, lane 4).

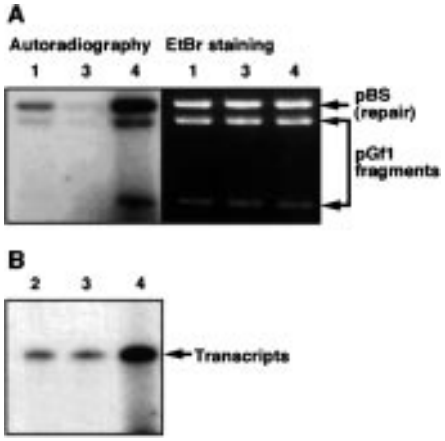

Figure 2. NER and RNA transcription activities in the optimized DNA repair–RNA transcription assay. UV-irradiated pBS (0.25 μg), non-damaged pBS (0.5 μg) and pGf1 (0.75 μg) were incubated with 100 μg GM01953C cell extract for 60 min at 30°C in the reaction mixture used in the repair assay (lane 1), transcription assay (lane 2), repair–transcription assay (unoptimized) (lane 3) and repair–transcription assay (optimized) (lane 4). The detailed conditions are described in Materials and Methods. For DNA repair (**A**), [α-^{32}P]dATP was added to the repair assay and repair–transcription assay (unoptimized) and [α-^{32}P]dGTP to the repair–transcription assay (optimized). For RNA transcription (**B**), [α-^{32}P]CTP was included in the transcription assay and repair–transcription assay (unoptimized) and [α-^{32}P]UTP in the repair–transcription assay (optimized). ^{32}P activity was visualized as described in Materials and Methods

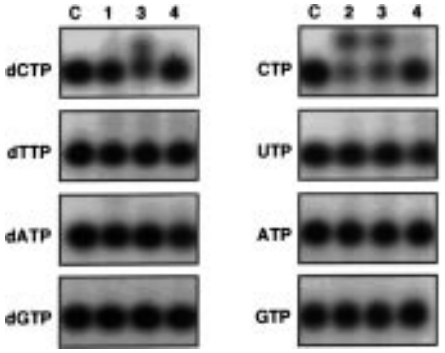

Figure 3. Stability of dNTPs and NTPs under cell-free assay conditions. The reactions were carried out with UV-irradiated pBS (0.25 μg), non-damaged pBS (0.5 μg) and pGf1 (0.75 μg) for 60 min at 30°C with 100 μg GM01953C extract in the reaction mixture of the repair assay (lane 1), transcription assay (lane 2), repair–transcription assay (unoptimized) (lane 3) and repair–transcription assay (optimized) (lane 4). The assay conditions were as described in Materials and Methods. The reactions also contained either 0.5 μCi [α-^{32}P]dATP, [α-^{32}P]dGTP, [α-^{32}P]dCTP, [α-^{32}P]dTTP, [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP or [α-^{32}P]UTP. ^{32}P activity was visualized as described in Materials and Methods. The control incubations without cell-free extracts are shown in lane C.

For transcription, no apparent difference in activity was found between the repair–transcription assay (unoptimized) and the slightly modified transcription assay of Dignam *et al*. (designated transcription assay; see Materials and Methods) (Fig. 2B, lanes 2 and 3). However, CTP was found to be unstable in both the transcription assay and repair–transcription assay (unoptimized) (Fig. 3, CTP, lanes 2 and 3). In the reaction with an increased CTP concentration (270 μM), such degradation was prevented (Fig. 3,

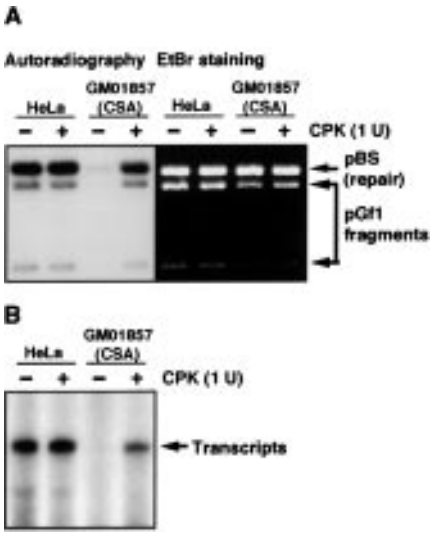

Figure 4. Effects of CPK on NER and RNA transcription activities. The repair–transcription assay (optimized) (see Materials and Methods) was used with UV-irradiated pBS (0.25 µg), non-damaged pBS (0.5 µg) and pGf1 (0.75 µg) for 60 min at 30°C with 100 µg protein extract prepared from either HeLa or CSA (GM01857) cells. Standard reactions contained 1 U CPK or not, as indicated. For NER (**A**), reactions contained [α-^{32}P]dGTP. For RNA transcription (**B**), [α-^{32}P]UTP was added to the reactions. ^{32}P activity was visualized as described in Materials and Methods.

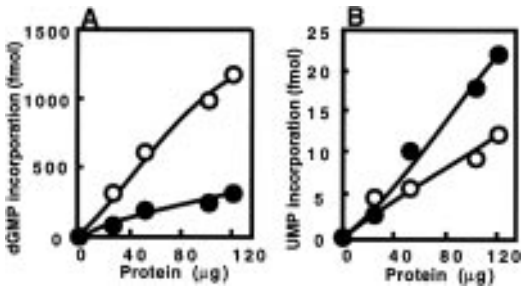

Figure 5. NER and RNA transcription by repair–transcription assay (optimized). The reactions were carried out with UV-irradiated pBS (0.25 µg), non-damaged pBS (0.5 µg) and pGf1 (0.75 µg) for 60 min at 30°C with various amounts of cell-free extract from either HeLa (○) or GM01953C (●) cells. The detailed assay conditions were as described in Materials and Methods. For NER (**A**), reactions contained [α-^{32}P]dGTP. ^{32}P activity incorporated into DNA was quantified to obtain the amount of dGMP incorporated into repair patches. For RNA transcription (**B**), [α-^{32}P]UTP was added to the reactions and ^{32}P activity in the RNase T1-resistant transcripts was quantified to obtain the amount of UMP incorporated into transcripts.

CTP, lane 4) and transcription activity was increased, as shown in Figure 2B (lane 4).

Thus, we have determined the optimal concentrations of dNTPs and NTPs to be: 1.2 mM ATP, 360 µM GTP, 180 µM UTP, 270 µM CTP, 600 µM dATP, 180 µM dGTP, 120 µM dTTP and 480 µM dCTP.

In the assay with lymphoblastoid cell extracts, NER and transcription activities required an added ATP-regenerating system. As shown in Figure 4A and B, <10% of NER as well as RNA transcription activity was found in reactions with CSA (GM01857) lymphoblastoid cell extracts when CPK was omitted from the reaction mixture, as compared with reactions in which CPK was added. We found that 40 U/ml CPK (1 U/25 µl reaction) were required to obtain maximum NER and transcription activities, although HeLa extracts did not require added CPK (Fig. 4).

In the optimized DNA repair–RNA transcription assay [designated repair–transcription assay (optimized); see Materials and Methods], we used 60 mM KCl, HEPES–KOH, pH 7.9, an ATP-regenerating system with 40 U/ml CPK and dNTPs and NTPs as described above. In this assay, about five and six times higher NER and RNA transcription activities were obtained, as compared with the activities obtained under the reaction conditions for the repair assay (Fig. 2A, lanes 1 and 4) and transcription assay (Fig. 2B, lanes 2 and 4) respectively.

Confirmation of the occurrence of NER and specific transcription initiation from AdMLP in the repair–transcription assay (optimized)

Consistent with the report by Coverley *et al.* (23), the addition of 20 µg/ml aphidicolin suppresses >95% of damage-specific ^{32}P incorporation in the assay and an antibody against RPA (70C) reduced incorporation by 50%. In addition, the 95% TFIIH-depleted extract exhibited only 40% of the control NER activity due to limited availability of TFIIH for NER; the addition of 0.83 µg (~1800 fmol) purified TFIIH restored NER activity to 70% of the non-depleted extract. These results confirmed that the ^{32}P incorporation detected in our assay was mainly due to NER.

For transcription, an antibody against TFIIE (2A1) or against TFIIH (3C9) reduced transcription activity by 70 and 40% respectively and negligible amounts of RNase T1-resistant transcripts were generated from pΔGf1, which lacked AdMLP but contained the G-less cassette. Furthermore, transcription activity was reduced to 30% when the 95% TFIIH-depleted extract was employed. However, addition of 1800 fmol purified TFIIH, restored the activity to 50% of the non-depleted extract, although addition of >1800 fmol TFIIH did not further increase RNA transcription activity, possibly due to removal of other transcription factors, which interact with TFIIH, during depletion. Taken together, these results suggest that our assay measured the occurrence of TFIIH-dependent transcription initiation from AdMLP by Pol II.

Titration of NER and RNA transcription activity

NER as well as RNA transcription activities increased linearly as a function of amount of extract used, at least up to 120 µg protein (4.8 mg protein/ml; Fig. 5A and B), and both reactions continued up to 4 h (data not shown). NER and transcription activities increased linearly, at least up to 0.75 µg UV-irradiated DNA and 0.75 µg pGf1 with 100 µg HeLa extract, for 60 min incubation (data not shown).

Analysis of relationships between NER and RNA transcription

Using the repair–transcription assay (optimized), relationships between NER and RNA transcription were analyzed with 100 µg extracts for 60 min incubation. To investigate the effect of RNA transcription on NER, DNA repair activity was compared between the assay with pGf1 and pΔGf1. As shown in Figure 6A, RNA transcription did not have a major effect on NER. Similarly, the effect of NER on RNA transcription was analyzed by

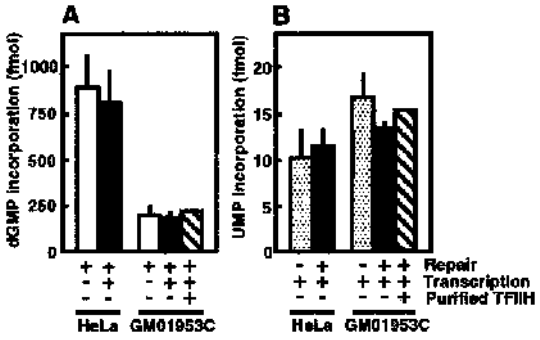

Figure 6. An analysis of the effect of RNA transcription on NER and of NER on RNA transcription. The reactions were carried out with 100 µg either HeLa or GM01953C extracts at 30°C for 60 min using the repair–transcription assay (optimized) (see Materials and Methods for details) with either [α-^{32}P]dGTP for NER (**A**) or [α-^{32}P]UTP for RNA transcription (**B**). The reactions for NER (A) also contained UV-irradiated pBS (0.25 µg), non-damaged pBS (0.5 µg) and either 0.75 µg pΔGf1 (white column) or 0.75 µg pGf1 (shadowed column). The reactions for RNA transcription by Pol II (B) contained pGf1 (0.75 µg) and either 0.75 µg non-damaged pBS (light shadowed column) or 0.25 µg UV-irradiated pBS as well as 0.5 µg non-damaged pBS (shadowed column). TFIIH (0.83 µg) was also added to the reaction with GM01953C extract with UV-irradiated pBS (0.25 µg), non-damaged pBS (0.5 µg) and 0.75 µg pGf1 (hatched column). The vertical bars indicate standard deviations.

performing reactions with pGf1 and either UV-irradiated pBS or non-damaged pBS. RNA transcription was not affected by NER, as shown in Figure 6B, although a reproducible but slight suppression of RNA transcription activity was found when GM01953C extracts were employed. These results suggest that NER is functionally independent of RNA transcription. Such independence may be a consequence of an excess of TFIIH activity over that required for NER and RNA transcription. In fact, in 100 µg HeLa and GM01953C extracts, ~2300 and 2400 fmol TFIIH respectively were found, as determined by Western blotting (data not shown). On the other hand, with 100 µg HeLa and GM01953C extracts 60 min incubation removed only 134 and 34 fmol UV-induced DNA lesions [calculated from the amount of incorporated dGMP into repair patches by assuming that the repair patch size is 29 nt (18) and that the patch contained an equal number of each dNMP] and produced 0.32 and 0.6 fmol RNase T1-resistant transcripts (calculated from the amount of UMP incorporation into RNase T1-resistant transcripts by dividing by 30, which is the number of uracil residues in the transcript).

In addition, supplementation with 1800 fmol purified TFIIH yielded only a negligible enhancement of both NER and RNA transcription activities (Fig. 6A and B). Furthermore, in the assay with the 95% TFIIH-depleted extract together with 1800 fmol purified TFIIH, no apparent competition between NER and RNA transcription was found (data not shown), indicating that the >1800 fmol TFIIH found in the 100 µg extract is sufficient to support NER and RNA transcription.

Therefore, we have concluded that NER is functionally independent of mRNA transcription in our assay system due to excess TFIIH activity in the extract.

DISCUSSION

In this report we have described a combined DNA repair–RNA transcription assay, which has required optimization of the relative concentrations of dNTP and NTP as well as CPK activity. During the cell-free reaction, nucleoside triphosphates were turned over (data not shown) and probably converted to nucleoside diphosphates. To regenerate dATP and ATP from corresponding nucleoside diphosphates, an ATP-regenerating system was added to the cell-free reactions. As described in this report, the assay with lymphoblastoid cell extract requires at least 40 U/ml CPK. Other nucleoside diphosphates may be regenerated by an endogenous enzyme, presumably nucleoside diphosphokinase (38), in cell-free extracts. This enzyme has a high K_m for dCDP and CDP relative to that for the other nucleoside diphosphates (38). Thus, dCDP and CDP tended to accumulate during the cell-free assay if the relative concentrations of dNTPs and NTPs are not optimized. Thus, optimization of the cell-free DNA repair–RNA transcription assay required adequate and balanced amounts of nucleoside triphosphates. We have determined the optimal concentrations for dNTPs and NTPs, based mainly upon the K_m of nucleoside diphosphokinase for nucleoside diphosphates (38).

In the repair–transcription assay (optimized), NER and RNA transcription activities were increased about five to six times when compared with the activity obtained in the repair assay and transcription assay. In this optimized assay, no apparent competition for TFIIH between NER and RNA transcription was found. Furthermore, addition of purified TFIIH to the assay did not significantly increase NER or RNA transcription activities. These results indicate that the cell-free extracts contain a sufficient amount of TFIIH to support both NER and RNA transcription. Thus, it can be considered that NER is functionally independent of RNA transcription initiation despite the fact that both processes are using the same factor. However, the sharing may provide certain survival advantages, since the dual role of TFIIH is conserved among eukaryotes, including yeast (4) and mammalian cells (1,3).

In *E.coli*, the NER enzymes UvrA and UvrB are induced to higher levels following the introduction of DNA damage, leading to modestly enhanced repair capacity (39), while mammalian cells seem to maintain a high NER capacity constitutively. In fact, we have confirmed significant amounts of NER activity in the extracts. As demonstrated by Donahue *et al.* (33), DNA damage on the transcribed strand of an actively transcribed gene can cause termination of RNA synthesis by stalling of Pol II. Thus, a single DNA lesion can abolish mRNA synthesis and this could be lethal if the damage in an essential gene is not repaired. The risk of termination of transcription may be reduced by maintaining high NER activity. On the other hand, there are sometimes very low levels of unscheduled DNA synthesis in cultured cells (see for example 27), so most of the time, NER may be considered to be an infrequent event relative to RNA transcription initiation. By using TFIIH, which is a factor with a housekeeping function, in NER, cells would be able to maintain high NER capacity regardless of the immediate requirement for NER activity and thus facilitate an efficient response to elevated levels of DNA damage. In this regard, the possible presence of two distinct forms of TFIIH, such as for transcription initiation and for NER, has been suggested (40). However, *in vitro* reconstitution experiments with purified factors clearly demonstrate that NER and RNA transcription initiation require only one form of TFIIH, since TFIIH which is active for RNA transcription initiation (29) is also active for NER (24). NER also shares RPA, PCNA, DNA polymerases and DNA ligase with the process of DNA repli-

cation. Such sharing of factors between NER and replication may have a similar physiological relevance to that of TFIIH.

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