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Mechanism of Release and Fate of Excised Oligonucleotides during Nucleotide Excision Repair*[S]

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Background: The fate of oligonucleotides released during nucleotide excision repair is unknown.
Results: Damage-containing oligonucleotides are released from duplex DNA in complex with TFIIH but then slowly dissociate and bind RPA or are degraded by nucleases.
Conclusion: Excised oligonucleotides form complexes with TFIIH and RPA during excision repair.
Significance: Oligonucleotides released during nucleotide excision repair may function in the cellular response to DNA damage.

A wide range of environmental and carcinogenic agents form bulky lesions on DNA that are removed from the human genome in the form of short, ~30-nucleotide oligonucleotides by the process of nucleotide excision repair. Although significant insights have been made regarding the mechanisms of damage recognition, dual incisions, and repair resynthesis during nucleotide excision repair, the fate of the dual incision/excision product is unknown. Using excision assays with both mammalian cell-free extract and purified proteins, we unexpectedly discovered that lesion-containing oligonucleotides are released from duplex DNA in complex with the general transcription and repair factor, Transcription Factor IIH (TFIIH). Release of excision products from TFIIH requires ATP but not ATP hydrolysis, and release occurs slowly, with a $t_{1/2}$ of 3.3 h. Excised oligonucleotides released from TFIIH then become bound by the single-stranded binding protein Replication Protein A or are targeted by cellular nucleases. These results provide a mechanism for release and an understanding of the initial fate of excised oligonucleotides during nucleotide excision repair.

In humans nucleotide excision repair is accomplished by dual incisions at 20 ± 5 phosphodiester bonds 5′ and 6 ± 3 phosphodiester bonds 3′ to the lesion to release the damaged base(s) in the form of a 24–32-nucleotide-long oligonucleotide (1–3) that has been referred to as "canonical ~30-nt excision product"[2] and the resulting single-stranded gap as the "canonical (30-nt) nucleotide excision repair gap" (4). The resulting ~30-nt gap is filled in by DNA polymerases without significant amount of nick translation or gap enlargement to produce the ~30-nt repair patch both *in vitro* (5) and *in vivo* (6, 7). The dual incisions releasing the ~30-nt excision product are accomplished by the combined actions of six repair factors, RPA, XPA, XPC, TFIIH, XPG, and XPF-ERCC1 (8–10). The basic mechanism of mammalian excision repair, including damage recognition, dual incisions, and repair synthesis to fill in the ~30-nt gap, have been investigated in considerable detail (11–14). However, no concerted attempt has been made to determine the fate of the excised oligonucleotide. In light of reports that defects in endo/exonuclease activities are associated with several human diseases including certain autoimmune disorders (15–19), it is conceivable that the canonical ~30-nt excision product plays a role in the cellular response to DNA damage and that improper processing of the excised oligonucleotide may contribute to the pathogenesis of certain human diseases precipitated or exacerbated by agents that damage DNA.

In this report, we therefore examined the fate of the excised oligonucleotides during nucleotide excision repair both with mammalian cell-free extracts and with purified human excision repair factors. Surprisingly, we find that the excised oligonucleotide is released in a complex with the transcription/repair factor TFIIH and that it dissociates from TFIIH in an ATP-dependent manner at a very slow rate, ultimately becoming targeted by cellular nucleases and/or bound by RPA. These results clarify an important step in the mechanism of excision repair and raise the possibility that the canonical 30-nt excision product may be involved as a signal for coordinating cellular responses to DNA damage.

EXPERIMENTAL PROCEDURES

Excision Repair Assay—Internally ^{32}P-labeled DNA substrate (140 bp) containing a single (6-4) UV photoproduct was prepared by annealing and ligating six oligonucleotides, as previously described (20). The oligonucleotide containing the (6-4) photoproduct was prepared by the Synthetic Organic Chemistry Core at the University of Texas Medical Branch (Galveston, TX) and subsequently radiolabeled with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs). In experiments with biotinylated substrate immobilized on Dynabeads M-280 streptavidin (Invitrogen), a 5′-biotinylated oligonucleotide was

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[2] The abbreviations used are: nt, nucleotide(s); TFIIH, Transcription Factor II H; RPA, Replication Protein A; CFE, cell-free extract; IP, immunoprecipitation; ssDNA, single-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate); AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate).

used in place of the corresponding nonbiotinylated oligomer of identical sequence. Immobilization of the DNA was done according to the manufacturer's recommendations (Invitrogen). Sequences of the oligonucleotides are available upon request. Standard excision assays involved incubation of 12.5 fmol of substrate in a 25-μl reaction containing 3 mg/ml CHO (AA8) or HeLa cell-free extract (CFE), 2 mM ATP, and 50 nM recombinant human RPA, in a final buffer containing 23 mM HEPES-KOH (pH 7.9), 44 mM KCl, 2.5 mM $MgCl_2$, 2.5% glycerol, 0.04 mM EDTA, and 0.2 mM DTT. CHO and HeLa CFEs were prepared as described (20). Mock and RPA-depleted HeLa CFE was prepared by incubating 150 μg of HeLa CFE three times for 1 h at 4 °C with either 10 μg of normal mouse IgG (Santa Cruz sc-2025) or anti-RPA34 antibody (Calbiochem NA18) bound to recombinant protein A/G PLUS-agarose (Santa Cruz). Recombinant RPA was expressed in BL21 (DE3) cells and purified as described (21, 22). Excision assays with the core excision repair factors (XPA, XPC, XPF-ERCC1, XPG, TFIIH, and RPA) were performed using standard approaches (20). At the indicated time points, the excision reactions were stopped by addition of SDS (0.34%) and proteinase K (20 μg). After incubation at 50–60 °C for 20 min, excision products were purified by phenol-chloroform extraction and then precipitated in ethanol. Excision products were separated on urea-containing DNA sequencing gels and then detected with a PhosphorImager. Radiolabeled oligonucleotides of known length were resolved on all gels as size markers. Excision repair activity was quantified using ImageQuant 5.2 software (GE Healthcare) by dividing the signal intensity from the small ~30-mer products by the total signal from both the full-length substrate and ~30-mer products.

Gel Filtration Chromatography—Excision reactions or purified excision products were loaded onto a 24-ml Superdex 200 gel filtration column (GE Healthcare) equilibrated in Buffer A (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12.5% glycerol, 12 mM $MgCl_2$, 0.5 mM EDTA, and 0.02% Nonidet P-40). Fractions (0.75–1.0 ml, depending on the experiment) were collected and either first deproteinized with proteinase K and phenol-chloroform extraction or directly precipitated in ethanol. Protein standards (ferritin, aldolase, and ovalbumin) were used as molecular size markers to calibrate the column.

Immunoprecipitation—Excision reactions (typically 25 μl) were diluted 4-fold with 100 μl of cold buffer B (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12.5% glycerol, 12 mM $MgCl_2$, 0.5 mM EDTA, and 0.1% Nonidet P-40) after adding the appropriate antibody (typically 0.5–2 μg) to the reaction. The reactions were rotated for 1–2 h at 4 °C and then incubated for 1 h with 15 μl of recombinant protein A/G PLUS-agarose (Santa Cruz). After washing twice with 0.5 ml of buffer B, immunoprecipitates (IPs) were either eluted with 100 mM glycine (pH 2.7) and then neutralized with 100 mM Tris-HCl (pH 8.8) or were deproteinized by incubation with SDS and proteinase K as described above. Antibodies used for immunoprecipitation included anti-mouse IgG (sc-2025), anti-rabbit IgG (sc-2027), anti-XPB (sc-293), anti-XPA (sc-28353), anti-p62 (sc-292), and anti-XPC (sc-74410) from Santa Cruz and anti-RPA34 (NA18) from Calbiochem.

Assays measuring the release of excision products from TFIIH were performed by first immunoprecipitating TFIIH from a 60-min, 100-μl excision reaction with CHO CFE and immobilized, radiolabeled substrate DNA. After washing the immunoprecipitate three times with 0.5 ml of cold buffer B and once with cold buffer C (30 mM HEPES-KOH, pH 7.9, 40 mM KCl, 3.2 mM $MgCl_2$), immunoprecipitates were resuspended in buffer C. ATP and analogs were added to a final concentration of 2 mM, as indicated. RPA was added at 50 nM, as indicated. Immunoprecipitates were dispensed into 50-μl aliquots and then incubated at 30 °C for the indicated lengths of time. The reactions were centrifuged (3000 × *g* for 30 s) to separate the unbound, released material from the bead-bound immunoprecipitate. After deproteinization, phenol-chloroform extraction, and ethanol precipitation, purified DNAs were resolved on denaturing gels. The percentage of excision product released from TFIIH was quantified by dividing the excision product signal from the unbound fraction by the total excision product signal in the unbound and unbound fractions.

Immunoblotting—Proteins in CFE, excision reactions, and immunoprecipitates were separated by SDS-PAGE and transferred to Hybond ECL membranes (GE Healthcare). Immunoblot detection of most of the proteins involved the use of the same antibody that was used for IP. RPA and XPA were detected with antibodies from Bethyl (A300–241A) and Santa Cruz (sc-853), respectively. Chemiluminescent detection of proteins was done with either Western blotting luminol reagent (sc-2048; Santa Cruz) or ECL Prime reagent (GE Healthcare).

RESULTS

Excision Repair Products Are Partially Degraded in Cell-free Extracts—To examine the fate of the canonical ~30-nt excision product generated by mammalian nucleotide excision repair, we prepared a (6-4) photoproduct-containing DNA substrate and incubated it with CHO cell-free extract that is known to possess robust excision nuclease activity (20). As shown in Fig. 1*A*, within 15 min a significant amount of 30-nt excision product (24–32 nt) was detectable. Excision products continued to accumulate until ~2 h, at which point (~40% of the photoproduct excised) the rate of excision declines (Fig. 1*B*) and the size of the excision products begins to decrease by several nucleotides (Fig. 1*A*, compare *lanes 3* and *5*). These results are in close agreement with earlier reports on degradation of the ~30-nt excision product in HeLa cell-free extracts (3) and *Xenopus* oocytes (2).

Although the nucleases that are responsible for degrading the excised oligonucleotide are not known, the change in size distribution of the excised oligonucleotides as a function of reaction time was striking. Further analysis of this phenomenon revealed that at early time points, the peak excision product size was ~26–28 nt, and at later time points this size decreased to 19–20 nt (Fig. 1*C*). We considered that this ~20-nt degradation product could be primarily due to a 3′ to 5′ nuclease that digests the 4–8 nt located 3′ to the (6-4) photoproduct of the canonical 24–32-nt excision product and then stalls at the adducted base. However, when we incubated a 5′-labeled, damage-free 27-mer single-stranded oligonucleotide in CHO cell-free extract, we similarly observed an intermediate ~20 nt in

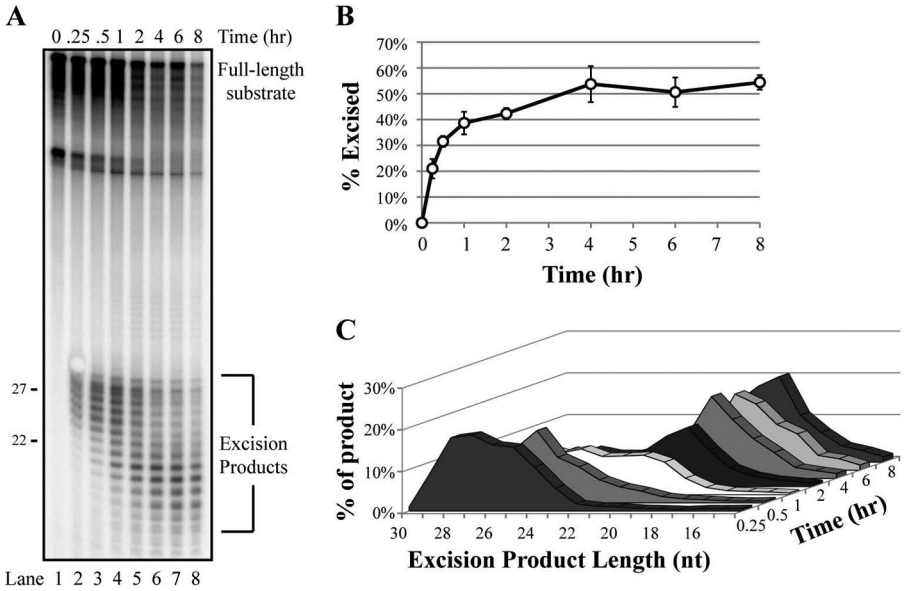

FIGURE 1. **Excised oligonucleotides become partially degraded in cell-free extract.** *A*, time course of excision repair in reactions with CHO AA8 cell-free extract. Locations of the full-length (6-4) photoproduct-containing substrate, the small (<30 nt) excision products, and oligonucleotide size makers (27 and 22 nt) are indicated. *B*, quantification of excision repair assays. The results indicate the averages and standard deviations from three independent experiments. *C*, distribution of excision product lengths during excision assay time course experiments. The results indicate the average from three independent experiments.

length that was apparently protected from nuclease digestion at the 3′ end of the oligonucleotide (supplemental Fig. S1). Thus, we conclude that the 19–20-nt oligonucleotides generated at later time points in excision reactions with a (6-4) photoproduct-containing substrate are not unique to single-stranded DNAs containing base adducts. These results further suggest that one or more cellular proteins may limit the degradation of the single-stranded DNA (ssDNA) excision product in cell-free extract.

Excision Repair Products Are Protein-bound—Using gel mobility shift assay (8) or DNA pull-down assay (23) to analyze the fate of the canonical 30-mer excision product, it was reported that the oligomer was released in a complex with a protein or was bound to a protein with high affinity for ssDNA shortly after release. However, a later study (24) employing gel filtration chromatography and an alternative method to detect the ~30-nt excision product concluded that the excision product was not associated with any of the six core repair factors.

To reconcile these conflicting conclusions regarding the status of the canonical 30-mer, we performed an excision reaction with CHO cell-free extract and the (6-4) photoproduct substrate and analyzed the reaction products by gel filtration chromatography. When the DNA was isolated by proteinase K digestion followed by phenol-chloroform extraction, the unrepaired DNA eluted at a position corresponding to a molecular mass of ~200 kDa, consistent with the calculated molecular mass of a duplex of 140 bp (Fig. 2*A*), whereas the excision products mainly eluted in the included volume consistent with the predicted molecular mass of ~10 kDa. When the reaction mixture was separated by gel filtration without further processing, the 140-bp substrate and the excision product were again separated; however, under this condition the unprocessed substrate was mostly in the void volume, consistent with its being associated with repair factors in preincision complexes and/or being bound nonspecifically to DNA-binding proteins present in whole cell-free extract. Importantly, the 30-mer eluted at positions corresponding to molecular masses of 150 kDa to greater than 400 kDa, consistent with the notion that the excision product is released either bound to one or more of the six repair factors (all 6 of which have DNA binding activity) or was bound to a repair factor or another DNA-binding protein present in the cell extract after release from the gapped duplex (Fig. 2, *B* and *C*). To differentiate between these two mechanisms, we performed the experiments described below.

Excision Repair Products of Discrete Sizes Associate with TFIIH and RPA in Cell-free Extract—In a previous study in which the canonical 30-mer was detected in DNA-protein complexes (23), it was speculated that the protein component might be XPC based on high affinity of this protein to ssDNA. To identify the protein(s) bound to the excised oligonucleotide, we performed an excision reaction with cell-free extract and then immunoprecipitated the various repair factors from the reaction with specific antibodies. As shown in Fig. 3*A*, we were able to efficiently immunoprecipitate RPA, XPA, XPC, and TFIIH (p62 and XPB) from the excision reaction. Importantly, under our reaction conditions each of the immunoprecipitates was free of the other excision repair proteins. It should be noted that because p62 and XPB are components of the multisubunit transcription/repair factor TFIIH, XPB was immunoprecipitated with both anti-p62 and anti-XPB antibodies.

When we analyzed the immunoprecipitates for DNA content, we observed that the canonical 30-mer was associated with only two factors, RPA and TFIIH (Fig. 3*A*). RPA is the major single-stranded DNA-binding protein in mammalian cells (25), and it plays multiple functions in nucleotide excision repair, including damage recognition, coordination and specification of nuclease activity, and repair resynthesis (11–13). TFIIH similarly functions at multiple steps during excision

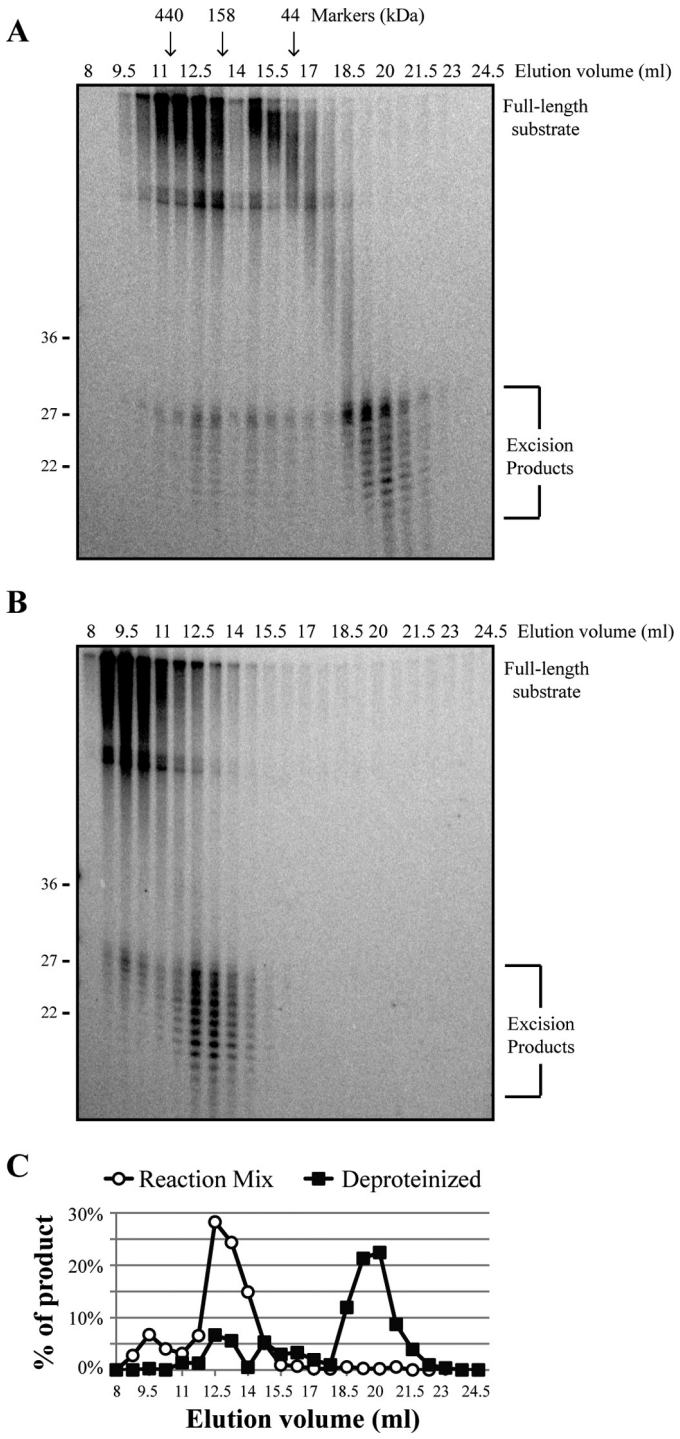

FIGURE 2. **The excised oligonucleotides are protein-bound.** *A*, products of an excision repair reaction (50 μl, 60 min) with cell-free extract were deproteinized as described under "Experimental Procedures" and analyzed by gel filtration chromatography on a Superdex 200 column. The fractions were then electrophoresed in a denaturing urea gel. Locations of the gel filtration protein standard size markers (ferritin, aldolase, and ovalbumin) are indicated. *B*, excision repair reaction mixture (50 μl, 60 min) containing both protein and DNA were directly analyzed by gel filtration chromatography. *C*, excision products from each of the fractions in *A* and *B* were quantified as a percentage of the total excision product signal across the column for each experiment and plotted. *Reaction Mix* indicates direct loading onto the column, and *Deproteinized* indicates deproteinization prior to gel filtration chromatography.

repair, including high specificity damage recognition by kinetic proofreading (26) and unwinding the DNA around the lesion by the XPD helicase in the 5′ to 3′ direction with respect to the damaged strand (27) to provide junction cutting of the repair bubble by the XPG and XPF nucleases. Remarkably, in Fig. 3*A*, the distribution of excision product lengths in the RPA and p62/XPB immunoprecipitates appears to be different, with the excision products associated with RPA being more distributed in length in comparison with the larger, more restricted oligomer length in the TFIIH-bound material.

To determine the extent of excision product association with TFIIH and RPA, we examined the relative amounts of the 30-mer in excision assays in which most of the target repair protein was depleted from the reaction mixture by immunoprecipitation. The results are shown in Fig. 3*B*. The most striking finding was that depletion of TFIIH with XPB and to a slightly lesser extent with p62 antibody resulted in the removal of the larger (25–30-nt) excision products. Quantification of the relative amount of 25–30-nt oligomers removed upon TFIIH immunodepletion indicated that between 60 and 80% of the excised "canonical 30-mer" was associated with TFIIH and that ~15–20% was associated with RPA (Fig. 3*C*).

To further examine the apparent difference in excision product length distributions of the oligomers associated with TFIIH and RPA, we repeated the gel filtration chromatography separation of the excised oligomer from an excision reaction carried out to 90 min, which provides sufficient time for considerable degradation of the excised oligomer in the extract (Fig. 1). As shown in Fig. 3*D*, this approach led to a clear separation of the 25–30-nt-long oligomers from the more degraded excision products (17–23 nt). Immunoblot analyses of these fractions showed approximate overlap of the larger excision products with TFIIH and the smaller oligomers with RPA. Importantly, immunoprecipitation of TFIIH and RPA from the two peak fractions verified that the majority of the larger, 25–30-nt (canonical) excision products immunoprecipitated with TFIIH, whereas the smaller, 17–23-nt oligomers associated only with RPA (Fig. 3*E*).

Because the 30-mer excision product is gradually degraded in cell-free extract, we next determined whether the lengths of the excision products associated with TFIIH and RPA changed as a function of reaction time. As shown in Fig. 3*F*, regardless of reaction time, the excision products that associated with TFIIH were only the longer (presumably the primary reaction product) oligomers 24–32 nt in length. At later time points, as the rate of excision declines while degradation of the canonical 30-mer continues (Fig. 3*F*, 4–6 h), there is less excision product associated with TFIIH. In contrast, the lengths of RPA-associated excision products at each time point are largely representative of the primary and secondary products in the whole reaction (input). These results further support the notion that the excised oligomers that associate with TFIIH and RPA represent distinct phases of the canonical 30-mer excision product.

Excision Products Generated in a Reconstituted Excision Reaction System Associate with TFIIH and RPA—All of the excision reactions performed so far were done with CHO cell-free extract as a source of excision factors. To determine whether excision products similarly become bound by TFIIH

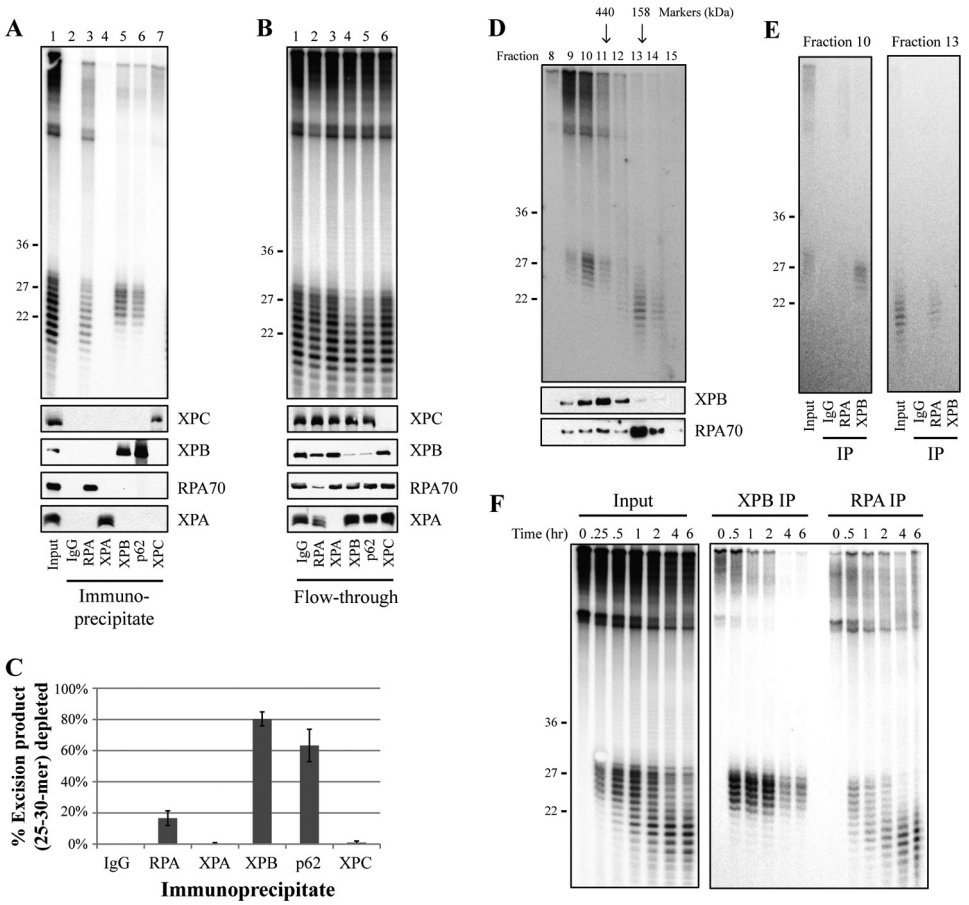

FIGURE 3. **The excised oligonucleotides associate with TFIIH and RPA.** *A*, the indicated proteins were immunoprecipitated from excision repair reactions (90 min) with specific antibodies. DNA from the immunoprecipitations were purified and then analyzed in a denaturing urea gel (*top panel*). Immunoprecipitates were also analyzed by SDS-PAGE and Western blotting. *B*, the unbound material (flow-through) from the immunoprecipitations in *A* were similarly processed and analyzed by denaturing gel electrophoresis and SDS-PAGE. *C*, quantification of the percentage of 25–30-mer oligonucleotides depleted from the excision assay immunoprecipitations in *B*. The results show the averages and standard deviations from three independent experiments. *D*, an excision repair reaction (100 μl, 90 min) was analyzed by gel filtration chromatography. Fractions were electrophoresed in both urea and SDS gels. *E*, fractions 10 and 13 from *D*, which represent peak fractions for two excision repair product species, were immunoprecipitated with either anti-mouse IgG or antibodies against RPA and XPB. Purified DNA from the IP was electrophoresed in a urea gel. *F*, time course experiments were performed, and then XPB and RPA were immunoprecipitated. DNA from the IPs were analyzed by urea-PAGE.

and RPA in a fully reconstituted system with purified human proteins, we repeated the excision reaction followed by gel filtration chromatography and immunoprecipitation experiments with the purified repair factors. As shown in Fig. 4*A*, when DNA was analyzed following deproteinization, most of the excised oligomers eluted in the included volume of the gel filtration column, consistent with a molecular mass of ~10 kDa. In contrast, and similar to that observed with cell-free extract, all of the excised oligomers eluted much earlier from the column when the excision reaction mixture was directly loaded on the column (Fig. 4, *B* and *C*). These results indicated that, similar to the reaction with the cell-free extract, all of the excised oligomers are in complex with protein in the reconstituted reaction. This was further confirmed by immunoprecipitation with TFIIH and RPA antibodies. As shown in Fig. 4*D*, the excised oligomers associated with both RPA and TFIIH, just as was observed in reactions with cell-free extract. However, we observed no evidence of degradation of the excision product when purified excision factors were employed. These results indicate that the degradation of the excised oligomers that occurs in cell-free extract is caused by one or more nucleases that are not present in the reconstituted system.

TFIIH and RPA Associate with Excision Products Released from Duplex DNA—To ascertain that TFIIH and RPA are associated with excision products that have been released from the canonical 30-nt gap, we carried out experiments with immobilized substrate and then analyzed the products. A schematic of the approach is shown in Fig. 5*A*: a 140-bp duplex containing 5′-biotin in one strand was added to streptavidin-coated magnetic beads and incubated with cell-free extracts, then the substrate (and gapped DNA with or without the "excised" oligomer) was removed from the reaction mixture with a magnet, and then the bound and unbound fractions were analyzed by gel electrophoresis/autoradiography. Consistent with a previous report (9), greater than 90% of the 30-mer excision product was released from the gapped duplex (Fig. 5*B*). Moreover, when we immunoprecipitated TFIIH and RPA from the soluble, unbound (not associated with duplex or gapped DNA) fraction, excision products of the expected lengths co-immunoprecipitated with RPA and TFIIH (Fig. 5*C*).

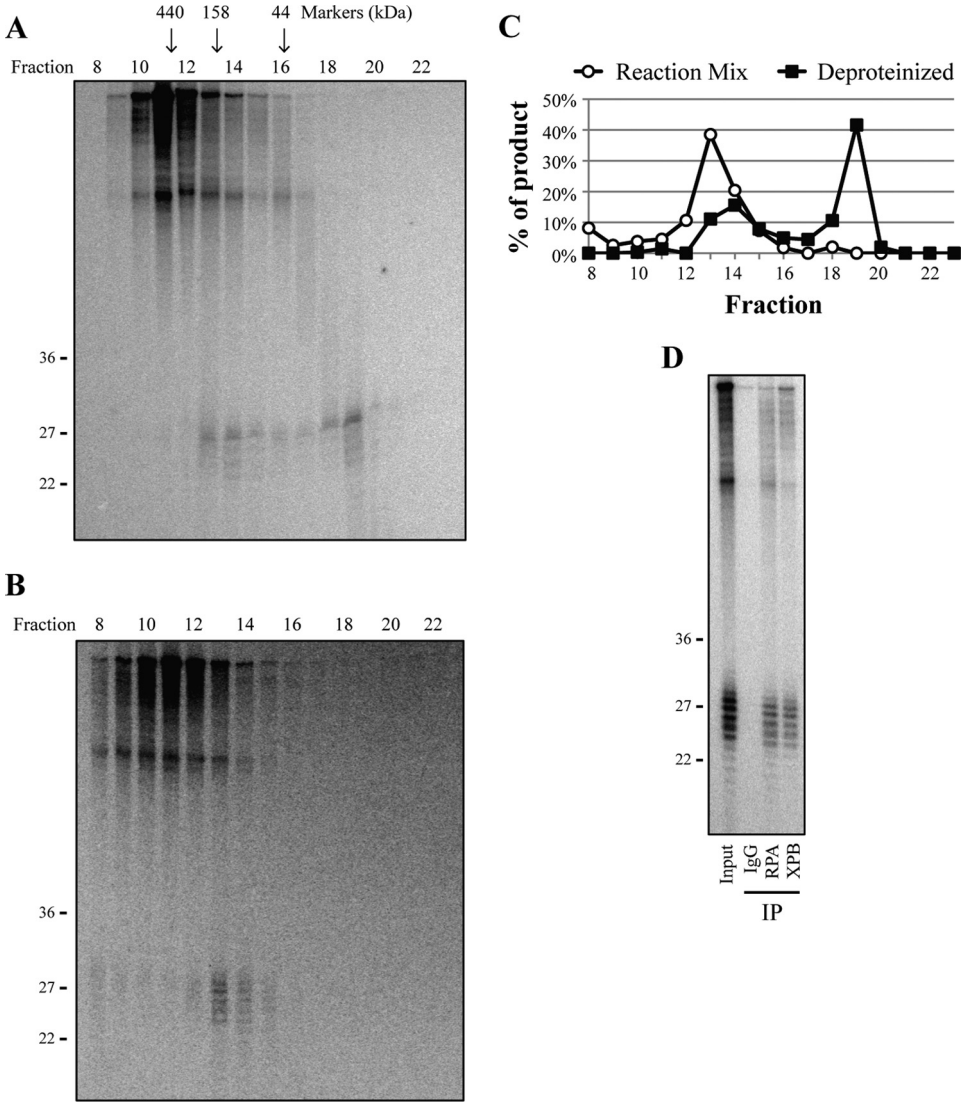

FIGURE 4. **Excised oligonucleotides generated with a reconstituted excision reaction associate with TFIIH and RPA.** *A*, deproteinized excision repair products from a reconstituted excision assay were analyzed by Superdex 200 chromatography and urea gel electrophoresis. *B*, a reconstituted repair reaction was directly fractionated by Superdex 200 chromatography followed by urea gel electrophoresis. *C*, quantification of excision product distribution in *A* and *B*. *Reaction Mix* indicates direct loading onto the column, and *Deproteinized* indicates deproteinization prior to gel filtration chromatography. *D*, a reconstituted reaction was subjected to immunoprecipitation with the indicated antibodies. DNA was purified and electrophoresed in a urea gel.

It is formally possible that the excised oligonucleotides were released from duplex (gapped) DNA as protein-free oligomers and then become bound by free TFIIH and RPA in the cell-free extract. To test for this eventuality, we performed an excision assay with the immobilized DNA substrate, removed the supernatant, deproteinized the excision products in the supernatant, and then reincubated the purified excised oligomers in reactions containing CHO cell-free extract. Interestingly, all of the excised oligomers became protein-bound and, in gel filtration chromatography, eluted in fractions corresponding to the elution peak of RPA (Fig. 5, *D* and *E*). Moreover, immunoprecipitation of RPA and TFIIH from the cell-free extract incubated with purified excision products revealed that the excised oligomers associate with RPA but not with TFIIH under this reaction condition (Fig. 5*F*). This result indicates that in standard reactions the association of dual incision products occurred by different means. As the most abundant high affinity ssDNA-binding protein in mammalian cells, RPA would be predicted to rapidly associate with any free ssDNAs that are released during excision repair. In contrast, the data in Fig. 5 taken as a whole most parsimoniously are consistent with the notion that the canonical 30-mer excision product is released from the excision gap in a complex with TFIIH. This model was further tested in subsequent experiments.

Release of Excised Canonical 30-mer from TFIIH Is ATP-dependent—The data presented so far are consistent with the notion that the excised oligomer is released in a complex with TFIIH and that it is subsequently released from TFIIH to be bound by RPA and/or degraded. To examine the release of the excision product from TFIIH, we performed excision reactions with immobilized substrate and cell-free extract and then immunoprecipitated TFIIH from the soluble material. After extensive washing of the immunoprecipitate, we incubated the TFIIH-excised oligomer complex in reaction buffer in the presence or absence of ATP. Then we separated the released oligomers from the TFIIH-bound excision products by

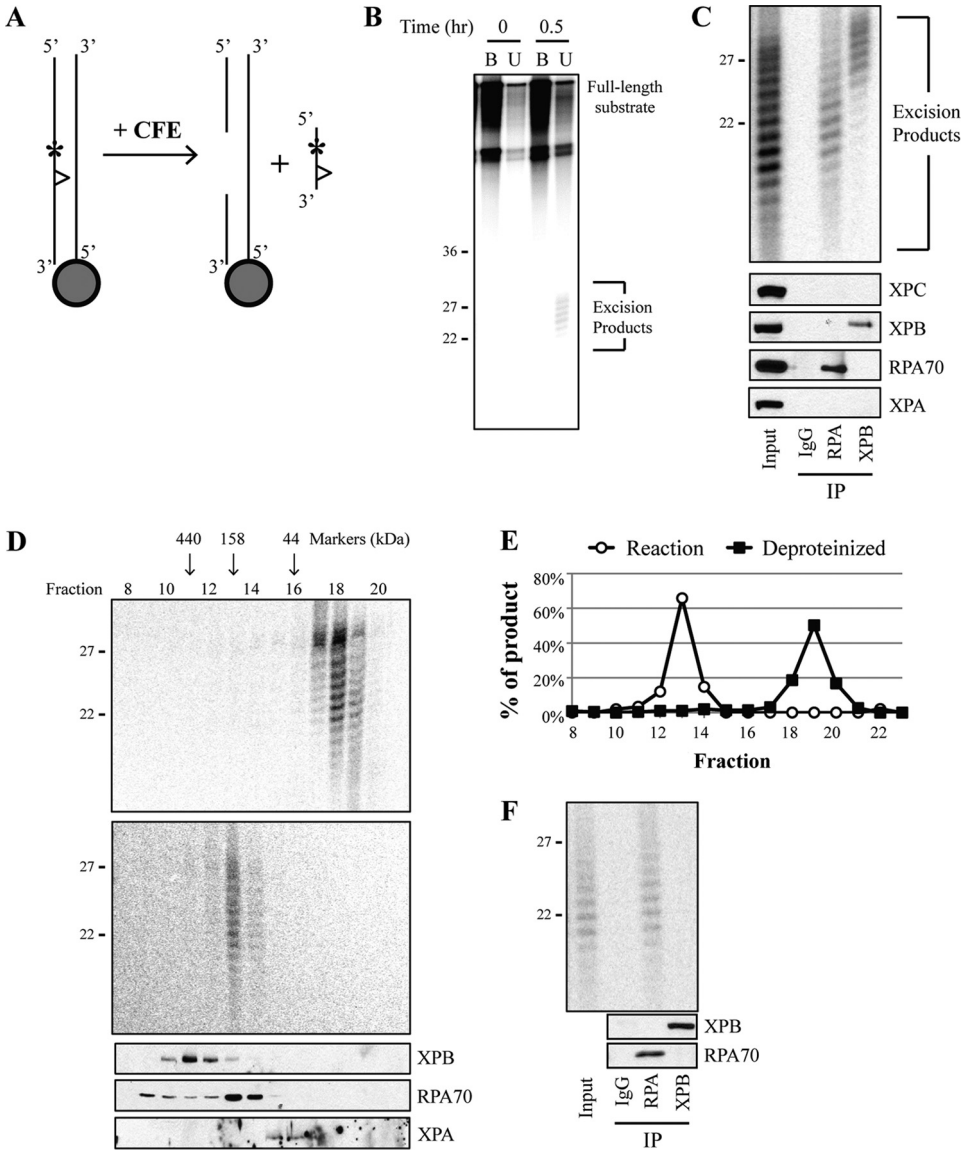

FIGURE 5. **TFIIH and RPA associate with excised oligonucleotides released from duplex DNA.** *A*, schematic for excision repair reaction with immobilized substrate DNA. Internally ^{32}P-labeled T-T (6-4) photoproduct-containing substrate was prepared as described under "Experimental Procedures" with one oligonucleotide containing a 5′-biotin, which allowed for immobilization on Dynabeads M280 streptavidin magnetic beads. An *asterisk* represents the radiolabel, and the *triangle* indicates the (6-4) photoproduct. *B*, DNA from excision assays with CHO CFE was collected on a magnet after the indicated periods of time (immediately or following 0.5 h of incubation). Bound (*lanes B*) and unbound (*lanes U*) DNA were purified and electrophoresed in a urea gel. *C*, excision products released from the immobilized DNA during a 90-min excision reaction were subjected to immunoprecipitation with the indicated antibodies and then analyzed by urea- and SDS-PAGE. *D*, excision products released from immobilized DNA during a 90-min excision reaction were purified and subjected to gel filtration chromatography and urea gel electrophoresis (*top panel*). A portion of the purified excision products were reincubated in a standard excision reaction for 30 min and then fractionated by gel filtration chromatography (*middle panel*). The fractions were analyzed by urea-PAGE (*top* and *middle panels*) and SDS-PAGE (*bottom panels*). *E*, quantification of excision product distribution in *D*. *Reaction* indicates direct loading of the excision products reincubated in CHO AA8 CFE onto the column, and *Deproteinized* indicates deproteinization prior to gel filtration chromatography. *F*, purified excision products reincubated in CHO CFE-containing reaction mixtures for 30 min were subjected to immunoprecipitation with the indicated antibodies.

centrifugation of the immunoprecipitate. As shown in Fig. 6 (*A* and *B*), the TFIIH excision product is remarkably stable, such that after an 8-h incubation there was no detectable release of the excised 30-mer from TFIIH (Fig. 6*A*, *top panel*). Importantly, in the presence of ATP, the canonical 30-mer was released, albeit at a slow rate (Fig. 6*A*, *bottom panel*). The slowly hydrolyzable and nonhydrolyzable ATP analogs ATPγS and AMP-PCP also supported release (Fig. 6*C*). In contrast, ADP and AMP failed to promote release of the excision product from TFIIH (Fig. 6*D*). These results indicate that ATP binding but not hydrolysis by TFIIH is necessary for dislodging the tightly bound 30-mer excision product. It must be noted, however, that the rate of release is very slow ($k_{off} = 6 \times 10^{-5}\ s^{-1}$ and $t_{1/2} =$ 3.3 h) as evident from the first order plot of the release reaction in Fig. 6*B*. A similar rate of release was observed when the TFIIH excision product complex was incubated with ATP in the presence of cell-free extract (data not shown). Thus, the TFIIH-30-mer complex is rather stable, and unless the release rate is accelerated by other factors *in vivo*, it may seriously interfere with cellular physiology.

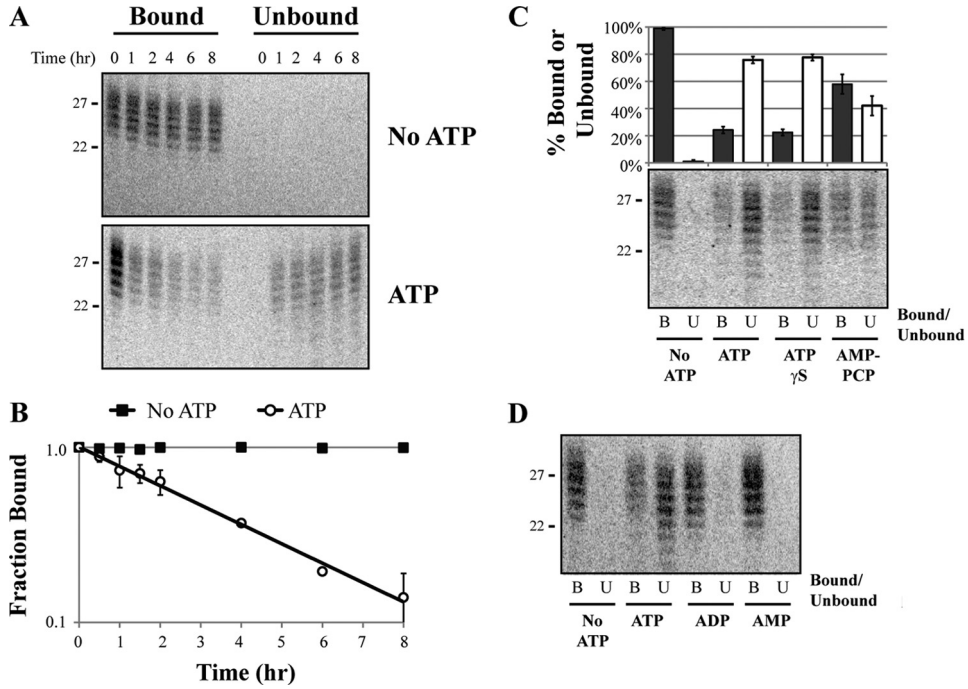

FIGURE 6. **The slow release of excision products from TFIIH requires ATP but not ATP hydrolysis.** *A*, a TFIIH immunoprecipitate from an excision reaction with CHO CFE and immobilized, radiolabeled substrate DNA was incubated in reaction buffer with either no ATP or with 2 mM ATP. At the indicated time points, the TFIIH immunoprecipitates were pelleted by centrifugation. Excision products that were released from TFIIH and that remained bound to TFIIH were purified and analyzed by denaturing urea-PAGE and phosphorimaging. *B*, quantification of results in *A*. The results show the averages and standard deviations from three independent experiments. *C*, TFIIH excision product immunoprecipitates described in *A* were incubated for 7 h in the presence of no ATP, ATP, ATPγ*S*, or AMP-PCP. The plotted data are the averages and standard deviations from three independent experiments. *D*, TFIIH excision product immunoprecipitates described in *A* were incubated for 7 h in the presence of no ATP, ATP, ADP, or AMP. *Lanes B*, bound DNA; *lanes U*, unbound DNA.

Effect of RPA on Release and Degradation of Canonical 30-mer Excision Product—We considered the possibility that RPA may facilitate the release of the excised oligomer and affect its rate of degradation. To test for this eventuality, we immunoprecipitated TFIIH following a standard excision reaction with cell-free extract and then incubated the immunoprecipitate in a buffer with or without ATP and in the absence or presence of RPA. We found that the canonical 30-mer is released only in the presence of ATP and that the extent of release is not affected by RPA (Fig. 7*A*). To determine whether under these conditions the released 30-mer bound RPA, following incubation of TFIIH-excision product + ATP + RPA, we immunoprecipitated RPA and analyzed the immunoprecipitate for DNA. As shown in Fig. 7*B*, the canonical 30-mer released from TFIIH is bound by RPA. The conclusion that emerges from these findings is that ATP binding by TFIIH promotes the release of the excised oligomer from TFIIH and the released oligonucleotide is bound by RPA.

Next, we wished to find out how RPA binding affected the degradation of the canonical 30-mer. To this end we depleted RPA from HeLa cell-free extract by immunoprecipitation (Fig. 7*C*). HeLa cell-free extract was used for these experiments because our RPA antibodies did not immunoprecipitate hamster RPA in CHO cell-free extract. Deproteinized and purified excision products associated with TFIIH immunoprecipitated from a standard excision reaction with CHO cell-free extract were then incubated with either the RPA-depleted HeLa cell-free extract or with RPA-depleted extract supplemented with >200-fold excess of recombinant RPA relative to excision product concentration. As shown in Fig. 7*D*, in the absence of RPA, the canonical 30-mer decreases in size and intensity with increasing reaction time. In contrast, the excised oligonucleotides are completely protected from degradation in the presence of recombinant RPA. Taken together, our data indicate that the excised 30-mer oligonucleotide is first released from duplex DNA in a complex with TFIIH. After the slow, ATP-dependent dissociation from TFIIH, the canonical 30-mer binds to RPA, which moderates its slow degradation by intracellular nucleases.

DISCUSSION

Human nucleotide excision repair encompasses several steps including damage recognition, excision of the damage by concerted dual incisions, release of the excised canonical 30-mer, and filling in of the excision gap by DNA polymerases and sealing by ligase (11–13). Of all these steps, the least characterized is the release of the canonical 30-mer and its ultimate fate. In Fig. 8 we present a human nucleotide excision repair model that incorporates the findings presented in this paper. Understanding the release and processing of the 30-mer is of significance from both a mechanistic standpoint for a comprehensive description of nucleotide excision repair as well as from a medical standpoint because of potential relevance of the excised oligonucleotide for autoimmune diseases.

Fate of Canonical 30-mer Excision Product—Several studies were carried out *in vivo* in both bacterial and mammalian cells on the fate of the cyclobutane pyrimidine dimers following nucleotide excision repair (28–33). These studies were carried

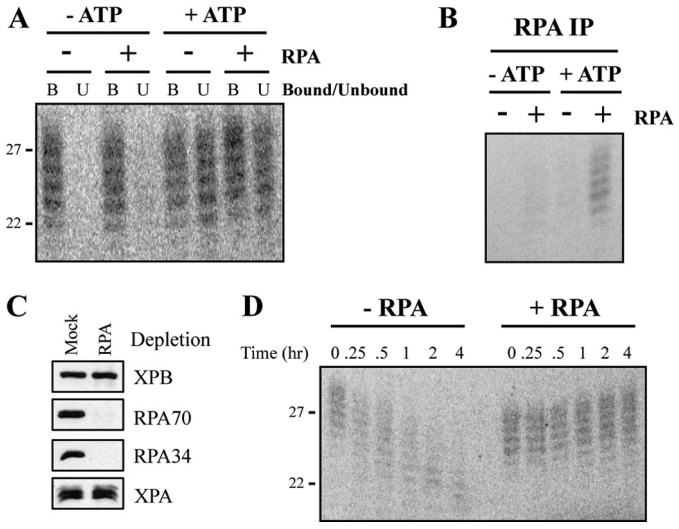

FIGURE 7. **RPA binds excision products released from TFIIH and limits excision product degradation.** *A*, TFIIH immunoprecipitates from an excision assay with CHO CFE and immobilized, radiolabeled substrate were incubated in reactions with or without RPA (50 nM) and ATP (2 mM). After the 7-h incubation, the TFIIH immunoprecipitates were pelleted by centrifugation, and the unbound and bound DNAs were purified and analyzed by urea-PAGE and phosphorimaging. *B*, a portion of the unbound fractions shown in *A* were subjected to a second round of immunoprecipitation with anti-RPA antibody. DNA associating with the RPA IP was analyzed by urea-PAGE and phosphorimaging. *C*, HeLa CFE was incubated with IgG or anti-RPA34 antibodies to yield mock and RPA-depleted CFE. Depleted CFE was analyzed by Western blotting with antibodies against the indicated proteins. *D*, excision products deproteinized and purified from a TFIIH immunoprecipitate from an excision reaction with CHO CFE and immobilized, radiolabeled DNA were incubated in new reactions containing RPA-depleted HeLa CFE supplemented or not with recombinant RPA (50 nM). At the indicated time points, the reactions were stopped, and the excision products were purified for analysis by denaturing urea-PAGE.

out prior to the discovery of excision by dual incision in both bacteria (34) and humans (1) and determined that in human cells the excised dimers were released in the form of oligonucleotides with an average length of 3.7 nucleotides (33). In view of our current knowledge about the excision mechanism, these oligonucleotides must have been processed excision products, but the processing pathway and the terminal product still are not known.

In fact, the only studies on the fate of the excised 30-mer were *in vitro* studies aimed at understanding its manner of release from the gapped duplex. The first report on the subject simply presented evidence that as determined by gel mobility shift assay, the excised canonical 30-mer was in complex with protein (8). A subsequent follow-up study using immobilized substrate suggested that XPC might be the repair factor bound to the canonical 30-mer during release from the dual incision complex (9). This latter suggestion was not based on additional experimental data but on the knowledge that the excision product was bound to a protein and extrapolating from the fact that of all six excision repair factors, XPC has the highest affinity for single-stranded DNA (35). However, subsequent work revealed that XPC is a molecular matchmaker and actually is not present in the dual incision complexes, preincision complexes 2 and 3 (36, 37), and hence could not be released in association with the canonical 30-mer. In contrast to these reports, a later study that employed gel filtration chromatography and an indirect method for detecting the excision product reported the excised 30-mer was released from the duplex free of protein and remained so in the reaction mixture (24). The experiments in the current paper, which employ gel filtration chromatography, immunoprecipitation, and a direct detection method for the canonical 30-mer, do in fact show that the excised oligomer is released in complex with protein and that that protein is TFIIH.

The observation that the excised oligomer is associated with TFIIH was a somewhat unexpected finding because so far the main role of TFIIH in nucleotide excision has been assumed to be in performing kinetic proofreading essential for high specificity damage recognition and as a part of this process to unwind the duplex around the damage to generate a "repair bubble" that enables XPG and XPF to bind and perform their endonucleolytic activities. Our findings in this paper, in conjunction with earlier work on TFIIH and repair, reveal that TFIIH actually participates in three key steps of human nucleotide excision repair: 1) damage recognition, 2) dual incision, and 3) release of the excised canonical 30-mer. The findings that the canonical 30-mer excision product is associated primarily with TFIIH and also (following ATP-dependent release from TFIIH) with RPA were somewhat surprising. In fact, there were some indications from a previous study that these two repair factors were unique vis-à-vis the excised oligomer: using a psoralen-thymine monoadduct as a substrate in UV-induced DNA-protein cross-link experiments with preincision/incision complexes, it was found that only the XPD subunit of TFIIH and RPA were cross-linked to psoralen, and it was concluded that these two repair factors must be in the vicinity of the damage in the repair complexes (38). This conclusion regarding the location of XPD in the repair complex has been confirmed with the present model expanded by x-ray crystallography (14).

It is also of interest that although there is no evolutionary relationship between the prokaryotic and eukaryotic excision repair systems (11) there are considerable similarities between the two systems brought about by convergent evolution, including ATP hydrolysis-dependent high specificity damage recognition and damage removal by concerted dual incisions. To these, one more similarity can now be added: the release of the excision product by helicase action following dual incision. In *Escherichia coli* the Uvr(A)BC excision nuclease carries out the dual incision where damage is recognized by UvrA-UvrB and the dual incision is made by UvrC in the UvrB-UvrC-DNA complex. Even though UvrB has translocase activity, it lacks a helicase function and following dual incisions the UvrC subunit dissociates from DNA leaving behind UvrB bound to the "excised 12-mer" in the "excision gap." The UvrB subunit, together with the bound 12-mer (like the TFIIH-30-mer complex), is released from excision gap by the UvrD 3′ to 5′ helicase to free the gap in preparation for repair resynthesis (39).

Processing of Canonical 30-mer Excision Product and Its Potential Pathophysiological Consequences—Several studies have suggested that improper processing of ssDNA fragments generated as intermediates during normal cellular metabolism or during processing of aberrant replication or recombination intermediates or during repair of double-strand breaks induced by ionizing radiation could lead to autoimmune diseases (16–18). Indeed, mutations in DNase1 (19) and the major 3′ to 5′ exonuclease Trex1 (40, 41) have been shown to give rise to

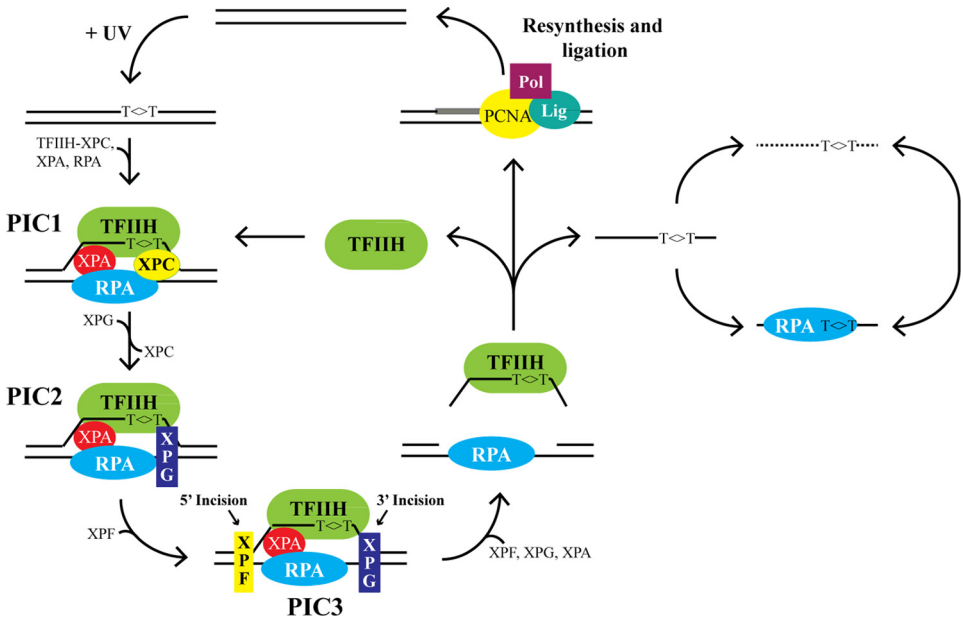

FIGURE 8. **Model for the mechanism of release and fate of excised oligonucleotides during nucleotide excision repair.** Double-stranded DNA exposed to UV light generates photoproducts in DNA, as shown by the T<>T dimer. The nucleotide excision repair factors TFIIH-XPC, XPA, and RPA assemble on the damaged DNA in a random and cooperative manner that involves the kinetic proofreading and helicase activities of TFIIH (*PIC1*, preincision complex 1). XPG enters and stabilizes the preincision complex, which coincides with XPC leaving the damaged DNA (*PIC2*, preincision complex 2). XPF associates with the other repair factors at the site of damage, followed by incision events by the XPF and XPG nucleases (*PIC3*, preincision complex 3). RPA remains on the gapped DNA to promote the subsequent repair resynthesis and ligation steps of nucleotide excision repair. The excision product is released in complex with TFIIH after the dual incision events. The ATP-dependent release of the excision product from TFIIH recycles TFIIH for use in additional repair events and leads to either oligonucleotide degradation and/or RPA binding to the excision product.

systemic lupus erythematosus and other autoimmune diseases. Two of the most common features of systemic lupus erythematosus are photosensitivity and production of anti-DNA antibodies. Often systemic lupus erythematosus first manifests itself after extensive sun exposure, and frequently this chronic disease is exacerbated by exposure to sunlight. Hence, it is conceivable that in genetically predisposed individuals, excessive sun exposure may lead to release of large amounts of excised 30-mers that exceed the capacity of the cell for rapid removal by endo- and exonucleases and results in formation of anti-DNA antibodies and related autoimmune reactions. This possibility, as well as the identity of the nucleases that degrade the canonical 30-mer, requires further investigation. We note that Trex1 does not appear to be involved in this process because the canonical 30-mer is degraded in wild-type and Trex1$^{-/-}$ mouse embryonic fibroblast cell-free extract with the same kinetics (supplemental Fig. S2). We also note that XPG and XPF do not appear to participate in degradation of the excised oligonucleotide because in the reconstituted excision system that contains both nucleases we do not detect significant degradation. Thus, the identity of the nuclease(s) that degrades the excision product remains unknown and will require a systematic analysis of mammalian endo- and exonucleases.

Finally, we note that the excised canonical 30-mer may have biological functions in the cell. Short, 5–10-nt ssDNA oligonucleotides generated during double-strand break processing have been shown to stimulate the checkpoint kinase ATM (42). Similarly, artificial ssDNA oligonucleotides with lengths comparable with those of nucleotide excision repair products activate p53 sequence-specific DNA binding (43). Thus, excised oligonucleotides generated during nucleotide excision repair may play important roles in the cellular response to DNA damage.

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