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Human DNA Repair Excision Nuclease

ANALYSIS OF THE ROLES OF THE SUBUNITS INVOLVED IN DUAL INCISIONS BY USING ANTI-XPG AND ANTI-ERCC1 ANTIBODIES*

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Human DNA repair excision nuclease removes DNA damage by incising on both sides of the lesion in a precise manner. The activity requires participation of 16–17 polypeptides. Of these, the XPF·ERCC1 complex and XPG were predicted to carry the nuclease active sites based on studies with the recombinant proteins and the yeast homologs of these proteins. Furthermore, recent work with model (undamaged) substrates have led to predictions of the roles of these proteins in incising 5′ or 3′ to the lesion. We have used damaged DNA substrates and antibodies to XPG and ERCC1 to test these predictions. Our results reveal that anti-XPG antibodies change the site of 3′ incision and at high concentration inhibit the 3′ incision without significantly affecting the 5′ incision, indicating that XPG makes the 3′ incision and further that under this condition 5′ incision can occur without 3′ incision. In contrast, anti-ERCC1 antibodies inhibit both the 3′ and 5′ incisions. Using a defined system for excision repair we also demonstrate that the 3′ incision can occur without the 5′ incision, leading us to conclude that under certain conditions the two incisions can occur independently.

In humans and yeast bulky DNA lesions are removed from DNA by an ATP-dependent multisubunit enzyme system which excises 27–29-nt[1]-long oligomers by incising the 3–5th phosphodiester bonds 3′ and the 22–24th phosphodiester bonds 5′ to the lesion (Huang *et al.*, 1992; Reardon *et al.*, 1993). Of the 16–17 polypeptides required for excision, studies with the yeast homologs of human proteins (Prakash *et al.*, 1993; Friedberg *et al.*, 1995) have proposed two strong candidates for making the dual incisions. RAD2 which is the homolog of XPG is a single-strand specific endonuclease (Habraken *et al.*, 1993). The RAD1·RAD10 complex which is the homolog of XPF·ERCC1 is an endonuclease with preference for single-stranded and supercoiled DNA (Tomkinson *et al.*, 1993; Sung *et al.*, 1993). Furthermore, human XPG protein has been also shown to have single-strand specific DNA endonuclease activity using recombinant protein from baculovirus system (O'Donovan *et al.*, 1994a; Habraken *et al.*, 1994a).

Recently, model DNA structures have been used with these proteins in an effort to identify which subunit in the excision nuclease complex makes which incision. Using partial duplexes with single-stranded tails or a flap structure as substrates and a truncated RAD2 protein, Harrington and Lieber (1994) concluded that RAD2 makes the 3′ incision and suggested that RAD1·RAD10 complex makes the 5′ nick. In apparent agreement with this it was found that purified recombinant XPG protein cleaved the junction of a duplex with single-stranded arms only in the strand with the 5′ single-stranded tail (O'Donovan *et al.*, 1994b) and the yeast RAD1·RAD10 complex cleaved only the strand with the 3′ single-stranded tail (Bardwell *et al.*, 1994).

However, two recent studies have raised some questions regarding the roles of these RAD and XP proteins in the dual incisions of the excinuclease. First, it was found that the RAD1 protein alone was capable of incising at the Holliday junction (Habraken *et al.*, 1994b), revealing that RAD1 contained the nuclease active site of the RAD1·RAD10 complex. Second, it was also found that RAD2 and XPG possessed 5′→3′ exonuclease activity (Habraken *et al.*, 1994c), leading to the suggestion that scission on the 3′ side was made by RAD1·RAD10 (XPF·ERCC1) followed by 5′ incision by RAD2 (XPG). Furthermore, it was proposed that RAD2 (XPG) digests the excised oligomer 5′ to 3′ to create a single-stranded gap necessary for the helicase functions of RAD3 (XPD) and RAD25 (XPB).

We wished to address the question using the excision assay of high specificity and sensitivity as well as incision assays and damaged DNA as substrate. Carrying out experiments with cell-free extracts (CFEs) and a defined system, and employing anti-XPG and anti-ERCC1 antibodies, lead us to conclude that in humans XPG makes the 3′ and XPF·ERCC1 makes the 5′ incision. Furthermore, we suggest that in human excinuclease the two incisions can occur independently under certain conditions.

EXPERIMENTAL PROCEDURES

Materials—HeLa S3 cells were from the stock of Lineberger Comprehensive Cancer Center (University of North Carolina). Xeroderma pigmentosum (XP) cell lines used in this study were: XP-A (GM02345B), XP-B (GM02252A), XP-C (GM02248B), XP-D (GM02253D), XP-E (CW3), XP-F (GM08437A), and XPG (AG08802 and GM03021A). The SV40-transformed XP2RO cell line (CW3) was obtained from R. Moses (Oregon State University, Corvallis, OR) via S. Linn (University of California, Berkeley). The XP-G lymphoblastoid cell line (AG08802) was a gift from K. H. Kraemer (National Institutes of Health, Bethesda, MD). The other human mutants were purchased from the National Institute of General Medical Sciences, Human Mutant Cell Repository (Coriell Institute, Camden, NJ). Chinese hamster ovary mutant cell lines, UV20(ERCC-1), UV41(ERCC-4=XP-F), and UV135(ERCC-5=XP-G), were obtained from the American Type Culture Collection Repository (Rockville, MD).

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[1] The abbreviations used are: nt, nucleotide(s); bp, base pair(s); CFE, cell-free extract; XP, xeroderma pigmentosum; MBP, maltose-binding protein.

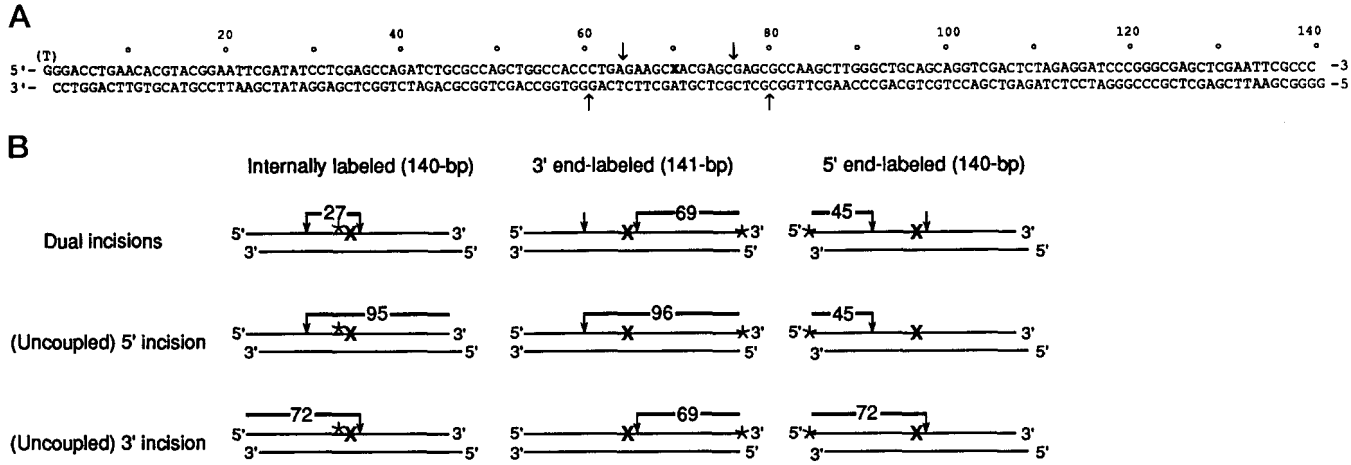

FIG. 1. **Substrates for the excision and incision assays.** *A*, internally labeled substrate was assembled from 6 oligomers after 5′ labeling of a cholesterol-containing oligomer (12-mer). For terminally (5′ or 3′) labeled substrates, two 140-nt-long single-stranded oligomers were used and the 5′ end of the 140-mer damaged strand contains T in place of G. *B*, internally labeled, 3′-labeled, and 5′-labeled substrates were used for excision assay, 3′ and 5′ incision assay, respectively. The predicted products after repair reaction with each substrate are shown schematically. The *X* and *asterisks* indicate the positions of cholesterol and the ^{32}P label, respectively.

Amylose resin, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. Klenow fragment of *Escherichia coli* DNA polymerase I and T4 DNA polymerase were purchased from Boehringer Mannheim and prestained protein markers were from Life Technologies, Inc.

XPG and ERCC1 Fusion Proteins—We constructed XPG and ERCC1 fusion proteins to produce antibodies. The plasmid pRAD2-synthetic carrying the human *XPG* cDNA (Scherly *et al.*, 1993) was kindly provided by S. G. Clarkson (University of Geneva Medical School). The *XPG* in this construct was subcloned into a pMAL-c2 vector (New England Biolabs) in-frame with *malE* to obtain pMAL-XPG which overproduces XPG fused to the maltose-binding protein (MBP). The construction of pMAL-ERCC1 has been described previously (Park and Sancar, 1993). The MBP-XPG and MBP-ERCC1 fusion proteins were overproduced in *E. coli* DR153/pMAL-XPG and DH5αF′*lacI*^{q}/pMAL-ERCC1, respectively. The proteins were purified through an amylose column as described by the manufacturer.

Antibody Production and Immunoblotting—Rabbit polyclonal anti-XPG and anti-ERCC1 antibodies were raised against MBP-XPG and MBP-ERCC1, respectively, by standard procedures. The anti-XPG antibodies were affinity-purified using a column containing the MBP-XPG fusion protein immobilized to AminoLink Plus Coupling Gel (Pierce). The purified antibodies were dialyzed against storage buffer (25 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 17% glycerol) and stored in small aliquots at −20 °C. Immunoblotting was performed by standard procedures.

Substrates—The substrate was a 140-bp duplex containing a cholesterol attached to a propanediol backbone instead of a nucleoside at position 70 of the "damaged" strand (Fig. 1*A*). In an extensive survey of commercially available modified bases and base analogs (including 2-amino-butyl-1,3-propanediol, biotin, and Texas red) we have found cholesterol-containing DNA (Clontech) to be the best substrate for human excinuclease. In contrast with other lesions such as thymine dimer, psoralen monoadduct or acetylaminofluorene-guanine adduct, this "lesion" is incorporated into a desired sequence by standard phosphoramidite chemistry and hence is readily available in large quantities.

Depending on the type of experiment performed we used internally labeled, 5′-labeled or 3′-labeled substrates (Fig. 1*B*). Internally labeled substrate was assembled from 6 oligonucleotides as shown in Fig. 1*A* (Huang *et al.*, 1994). The ^{32}P label was at the 6th phosphodiester bond 5′ to the lesion. The 5′-labeled substrate was prepared by labeling the 140-nt-long single-stranded oligomer with the lesion using [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by annealing to the complementary oligonucleotide. For 3′ end-labeling the two 140-nt oligomers were annealed and incubated with [α-^{32}P]dCTP, dATP, and Klenow fragment of *E. coli* DNA polymerase I. Filling-in the one nucleotide gap generates a duplex of 141 bp with blunt ends. The unmodified and cholesterol-containing oligomers were synthesized by Operon Biotechnologies and Midland Certified Reagents.

Repair Assays—CFEs prepared by the method of Manley *et al.* (1980) or the recently developed reconstituted system (Mu *et al.*, 1995) were used to measure excision (release of an oligomer containing damage by dual incisions) or incision (detection of one of the incision sites relative to the lesion using terminally labeled DNA). The excision assay was performed as described previously (Huang *et al.*, 1992, 1994). Briefly, the reaction mixture (25 μl) contained 32.5 mM Hepes-KOH, pH 7.9, 50 mM KCl, 4.4 mM $MgCl_2$, 0.15 mM EDTA, 0.4 mM dithiothreitol, 1.7% glycerol, 2 mM ATP, 100 μM of each dNTP, 200 μg/ml bovine serum albumin, ~1 nM internally labeled substrate, and 50 μg of CFE. The mixture was incubated at 30 °C for 45 min unless indicated otherwise and the products were deproteinized, ethanol-precipitated, and resolved on 10% polyacrylamide sequencing gels. The incision assay was conducted similarly except the substrate was terminally labeled and dNTPs were omitted. Excision assay with the reconstituted system was performed by mixing 5 highly purified fractions as described previously (Mu *et al.*, 1995): MBP-XPA (I), TFIIH/XPG (II), XPC (III), ERCC1·XPF (IV), and recombinant replication protein A (V). Quantitation of excision (oligomers in the 23–29-nt range) relative to the recovery of added substrate was determined by scanning autoradiographs with a Molecular Dynamics Computing Densitometer Series 300 instrument.

Analysis of the Excision Products—The location of cholesterol relative to the termini of the excised fragment was determined by using the 3′→5′ exonuclease activity of T4 DNA polymerase as described previously (Huang *et al.*, 1992; Svoboda *et al.*, 1993).

RESULTS

Specificity of Anti-XPG and Anti-ERCC1 Antibodies—Since the effect of antibodies played a central role in our analyses of the incision reactions, it was important to have high-specificity antibodies. In fact, antiserum raised against ERCC1 (α-ERCC1) was of high titer and gave clear signal without prior purification (Mu *et al.*, 1995). However, since the anti-XPG serum (α-XPG) showed high background, specific antibodies were obtained by affinity chromatography. Fig. 2*A* shows that of all of the XP cell lines tested only the two XP-G mutants lack the immunoreactive ~180-kDa protein which we identify as XPG in agreement with highly purified protein preparations (O'Donovan *et al.*, 1994a; Habraken *et al.*, 1994a). In one XPG mutant (GM03021A) with residual excision activity (Reardon *et al.*, 1993), a truncated XPG protein of ~140 kDa was reproducibly detected, although the signal was too faint in this particular experiment (*lane 9*). Similarly, α-ERCC1 revealed a specific band of ~40 kDa in all of XP cell lines tested (Fig. 2*B*) in agreement with previous reports that ERCC1 does not correspond to any XP gene (van Duin *et al.*, 1988) or protein (Reardon *et al.*, 1993). The variability in relative intensity may be real as in the case of XP-F with low intensity as has been reported (Biggerstaff *et al.*, 1993) or simply a reflection of the quality of extract. In any event, Fig. 2 reveals that our α-XPG

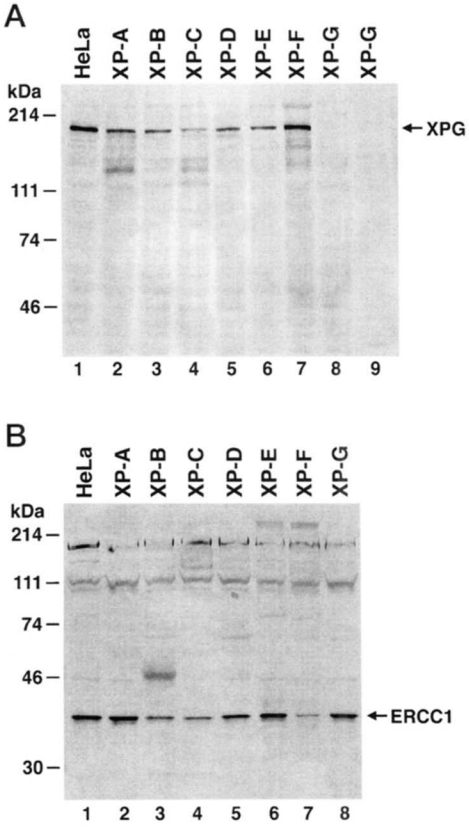

FIG. 2. **Specificity of anti-XPG and anti-ERCC1 antibodies.** CFEs (100 μg each) from HeLa (*lane 1*) and various XP cell lines (*lanes 2–9*) as described under "Experimental Procedures" were separated by 8% (*A*) or 10% (*B*) SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with α-XPG or α-ERCC1, respectively. In experiment *A*, XP-G CFE prepared from GM03021A (*lane 9*) in addition to AG08802 (*lane 8*) was used. The positions of prestained protein markers in kDa are indicated.

and α-ERCC1 are of sufficient specificity for use in functional assays.

Effects of Anti-XPG and Anti-ERCC1 Antibodies on Dual Incisions—To study the roles of XPG and XPF·ERCC1 complex in the dual incisions, we conducted excision assays in the presence of either α-XPG or α-ERCC1. Fig. 3*A* shows a unique effect of α-XPG on the excision reaction. At low concentration it enhances the excision of the 29-mer (7-fold) while having only mild inhibitory effect (33%) on the overall excision (*lane 4*); at higher concentration the 29-mer becomes a major excision product concomitant with more pronounced overall inhibition (67%) of 23–29-mer excision products (*lane 5*). Both effects can be reversed with appropriate amounts of the antigen (*lanes 6* and *7*). Excision in the presence of α-ERCC1 revealed only inhibition of excision by the antibodies (*lanes 8* and *9*, 87 and 94%, respectively) which was reversed by addition of recombinant ERCC1 (*lanes 10* and *11*). The unusual effect of α-XPG led us to investigate these findings in more detail.

Anti-XPG Antibodies Affect the Site of the 3′ Incision—The larger excision fragment induced by α-XPG could be due to a change in the 3′ or 5′ incision site or both. To differentiate among these possibilities we cut out from the gel the 27-mer major excision product and the 29-mer induced by α-XPG and treated the excised fragments with T4 DNA polymerase 3′→5′ exonuclease (Fig. 4). This activity is inhibited by lesions in DNA (Fuchs, 1984). The exonuclease treatment generates the same size fragment (26-mer) from both the 29-mer and the 27-mer (*lanes 5* and *7*, respectively). Thus, we conclude that α-XPG shifts the 3′ incision site without affecting the site of 5′ incision. Two possibilities were considered for this effect. Either XPG is at the 5′ incision site and when bound to antibodies the entire incision complex is displaced causing a change of incision site by XPF·ERCC1 located at the 3′ site, or XPG makes the 3′ incision and a subpopulation of the antibodies upon binding to XPG do not inhibit the incision reaction but displace the protein slightly such that the 5th phosphodiester bond which is normally attacked by XPG at a low frequency becomes a major target.

We reasoned that looking at the particular incision sites under α-XPG inhibitory conditions would help answer these questions. Indeed, higher resolution gels reveal that α-XPG, in addition to the two effects observed in Fig. 3*A*, also caused the appearance of a 95 nt-long fragment with this internally labeled substrate (Fig. 3*B*). This fragment corresponds to incision at the 25th phosphodiester bond 5′ to the lesion and hence represents an "uncoupled" 5′ incision[2] (see below). Our polyclonal antibodies must have at least two types of antibodies. One binds to XPG and changes its specificity; the other inhibits its function and causes the excinuclease to make only the 5′ incision. Taken together these data are consistent with XPG making the 3′ incision.

Analysis of XPG and XPF·ERCC1 Functions by the 3′ Incision Assay—In this assay terminally labeled substrate was incubated with the enzyme system and the activity was monitored by appearance of bands due to incision 5′ or 3′ to the lesion. Fig. 5 shows the result of such an assay with 3′-labeled substrate. HeLa CFE makes a major incision at the 3rd phosphodiester bond 3′ to the lesion, an infrequent incision at the 4th and a moderate level of incisions at the 2nd and 5th phosphodiester bonds 3′ to the lesion (*lane 2*). The incisions are specific to human excinuclease because the bands are absent from DNA treated with CFEs from XP-F (*lane 3*) and XP-G (*lane 4*) mutants and can be restored by mixing the two mutant extracts (*lane 5*). This conclusion is further supported by similar assays with Chinese hamster ovary excision repair mutants. UV20 (ERCC-1) (*lane 6*), UV41 (XP-F) (*lane 7*), and UV135 (XP-G) (*lane 8*) do not show 3′ incision consistent with lack of excision (Reardon *et al.*, 1993) and repair synthesis (O'Donovan and Wood, 1993) by these mutants. Furthermore, CFEs of ERCC-1 and ERCC-4 (XP-F) mutants fail to complement in the incision assay (*lane 9*), consistent with the conclusions that these two proteins are in a tight complex which does not exchange subunits readily *in vitro* (Reardon *et al.*, 1993). In contrast, CFEs of UV20 plus UV135 and UV41 plus UV135 do complement (*lanes 10* and *11*, respectively). In these latter two the 3′ incision occurs with about equal frequency at the 3rd and 5th phosphodiester bonds.

Having thus shown that the 3′ incision can be detected in this assay with 3′-labeled DNA, we wished to confirm the observations we made with the internally labeled substrate using the 3′ incision assay. Fig. 6 shows the results of such an assay. The α-XPG has two specific effects in addition to overall inhibition of 3′ incision (*lanes 5* and *6*, 71 and 88% inhibition, respectively): first, they reverse the frequency of incisions at the 3rd and 5th phosphodiester bonds 3′ to the lesion (*lane 5*, ratio of incisions is 1 to 1.7) compared to the control (*lane 1*, ratio of 1 to 0.4); second, they give rise to a band at position 96 (corresponding to position 95 in the 140-bp substrates) which can only be generated from a 5′ incision uncoupled from 3′ incision (Fig. 1*B*). These results are consistent with the data obtained with the internally labeled substrate. In contrast, α-ERCC1 inhibits 3′ incision entirely but does not produce the

[2] Uncoupled 5′ or 3′ incisions refer, respectively, to reactions in which the excinuclease makes the 5′ incision but fails to make the 3′ incision or vice versa.

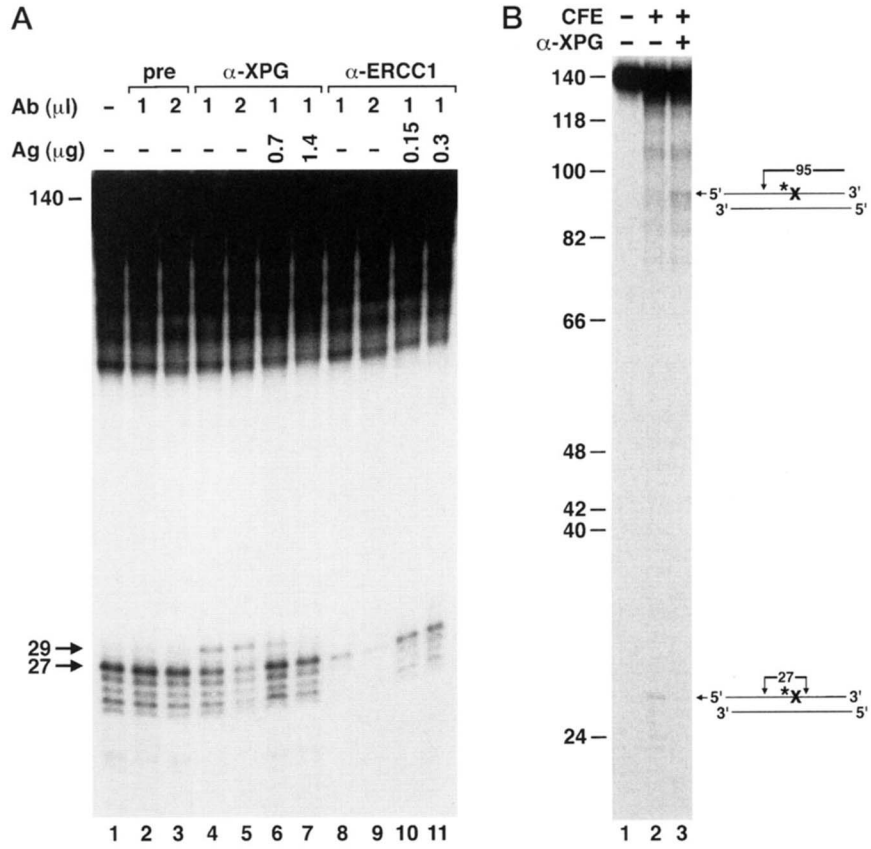

FIG. 3. **Effects of anti-XPG and anti-ERCC1 antibodies on dual incisions.** *A*, the indicated amounts of antibodies (*Ab*), preimmune, α-XPG, or α-ERCC1 were preincubated (for 10 min on ice) with HeLa CFE (50 μg) in the absence (*lanes 1–5, 8,* and *9*) or presence of antigen (*Ag*), MBP-XPG (*lanes 6* and *7*), or MBP-ERCC1 (*lanes 10* and *11*), and then added to the reaction mixtures containing the internally labeled substrate (140-bp). The excision products were resolved on a 10% polyacrylamide sequencing gel and the positions of the substrate (140-mer) and the products (27- and 29-mers) are indicated. *B*, the excision products were resolved on a 10% polyacrylamide sequencing gel for longer periods to increase the resolution of larger fragments. Two μl of α-XPG were used (*lane 3*) and the positions of DNA size markers (φX174/*Hin*fI) are indicated in the left margin. *Right panel*, schematic drawings of the incision pattern of human excinuclease on the internally labeled 140-bp substrate. In the absence of antibodies, 7.1% of the damage was excised as determined by the intensity of the bands.

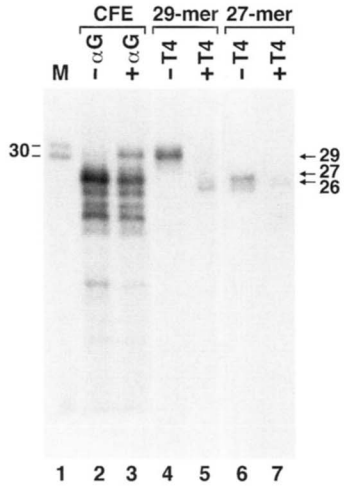

FIG. 4. **Perturbation of the 3′ incision site by anti-XPG antibodies.** After excision reaction with HeLa CFE in the presence of α-XPG, the products were separated on a 10% polyacrylamide sequencing gel, and the 27-mer major excision product and the 29-mer induced by α-XPG were cut out from the gel. The eluted products were resolved on a 10% polyacrylamide sequencing gel untreated (*lanes 4* and *6*) or after treatment with T4 DNA polymerase 3′→5′ exonuclease (*lanes 5* and *7*). For comparison, the products from excision reaction without (*lane 2*) or with (*lane 3*) α-XPG (1 μl) were also examined. The positions of the markers (30-mer) and the products (26-, 27-, and 29-mers) are indicated. The excised fragments containing a cholesterol molecule migrate 1 nt slower than unmodified DNA of the same length (see Fig. 6).

uncoupled 5′ incision, indicating that α-ERCC1 possibly inhibits 5′ incision as well. Further experiments were conducted with 5′-labeled substrate to ascertain our conclusions regarding the roles of XPG and XPF·ERCC1 on 5′ incision.

Incision Assay with 5′-Labeled DNA—In Fig. 7 we show that HeLa CFE makes several incisions centered around the incision at the 25th phosphodiester bond 5′ to the lesion (*lane 3*). These bands are not produced by XP-F (*lane 4*) or XP-G (*lane 5*) extracts and the activity is restored by mixing the two extracts (*lane 6*). Mutant Chinese hamster ovary cell lines UV20 (ERCC-1), UV41 (XP-F), and UV135 (XP-G) are also defective in 5′ incision (*lanes 7–9*), and the combination of UV20 + UV41 again fails to restore the activity (*lane 10*) but UV20 + UV135 and UV41 + UV135 combinations show complementation of the incision activity (*lanes 11* and *12*). These results taken together with those in Fig. 5 reveal that the lack of excision (Reardon *et al.*, 1993) and repair synthesis (O'Donovan and Wood, 1993) is due to the lack of both 3′ and 5′ incisions in the mutant cell lines. In addition, we could not detect any (uncoupled) 3′ and 5′ incisions in all other XP mutant CFEs used in this study.[3]

Having established a 5′ incision assay, we then investigated the effects of antibodies on the 5′ incision. Fig. 8 shows that α-XPG inhibits 5′ incision only moderately (*lanes 5* and *6*, 6 and 53% inhibition, respectively) compared to the control preimmune serum (*lanes 3* and *4*, 4 and 21%, respectively), whereas α-ERCC1 inhibits the 5′ incision as efficiently as the 3′ incision (*lanes 7* and *8*, 69 and 80%, respectively). Hence, we conclude that a subset of α-XPG inhibits the 3′ incision but not the 5′ incision, while α-ERCC1 inhibits both the 3′ and 5′ incisions. Our data up to this point strongly suggest that XPG makes the 3′ incision and XPF (in the form of XPF·ERCC1 complex) makes the 5′ incision and further that the 5′ incision can occur without the 3′ incision. The inhibition of both 5′ and 3′ incision by α-ERCC1 could be by two mechanisms. First, the 5′ incision is made initially and inhibition of this incision indirectly prevents 3′ incision; second, the XPF·ERCC1 complex is essential for the assembly of the excinuclease complex and prevention of binding of XPF·ERCC1 to the other subunits of the excinucle-

[3] T. Matsunaga and A. Sancar, unpublished results.

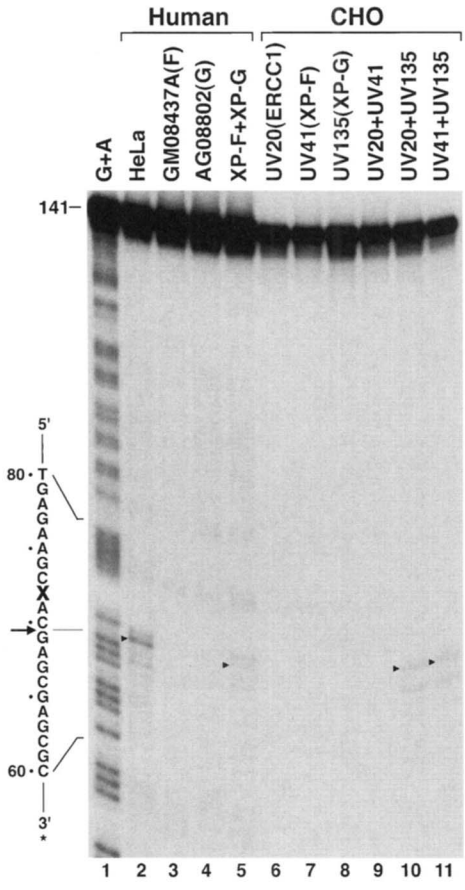

FIG. 5. **Incision assay with 3′-labeled substrate (3′ incision assay).** Fifty μg of HeLa CFE (*lane 2*) or mutant CFEs (*lanes 3, 4* and *6–8*) were incubated with 3′ end-labeled substrate at 30 °C for 60 min. In complementation reactions (*lanes 5* and *9–11*), 25 μg from each cell line was premixed and then added to the reaction mixture. The products were resolved by electrophoresis on a 10% polyacrylamide sequencing gel. *Lane 1* shows the Maxam-Gilbert reaction of G+A. The *arrow* and *triangles* indicate the major 3′ incision site. The position of the substrate (141-mer) is also indicated. As determined by the intensity of the bands relative to the total amount of the DNA, 9.3% of the total radioactivity was detected in the 67–70-nt region with HeLa CFE (*lane 2*), in contrast to 0.2–0.8% (background) with mutant CFEs (*lanes 3, 4,* and *6–8*). In complementation reactions (*lanes 5, 10,* and *11*), 4.8–5.6% of that was detected in this region (*i.e.* approximately 50–60% complementation). *CHO*, Chinese hamster ovary.

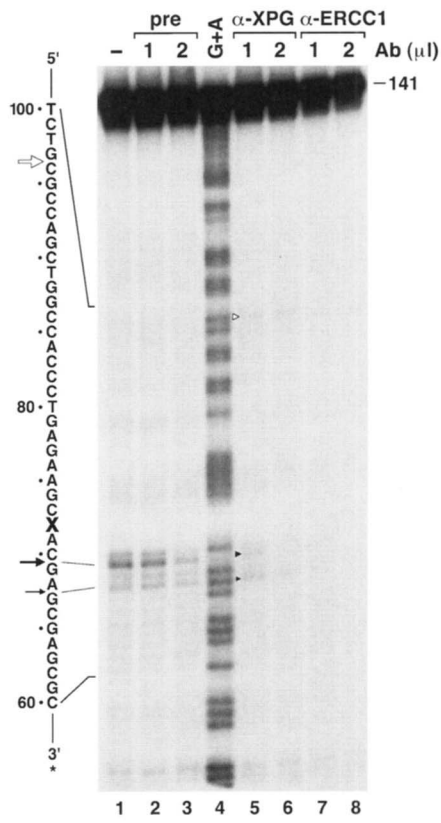

FIG. 6. **Effects of anti-XPG and anti-ERCC1 antibodies on the 3′ incision.** HeLa CFE (50 μg) was preincubated (for 10 min on ice) with the indicated amounts of antibodies (*Ab*), preimmune (*lanes 2* and *3*), α-XPG (*lanes 5* and *6*), or α-ERCC1 (*lanes 7* and *8*), and then added to the reaction mixture containing 3′ end-labeled substrate (30 °C, 60 min). The products were resolved by electrophoresis on a 10% polyacrylamide sequencing gel. *Lane 4* shows the Maxam-Gilbert reaction of G+A. The *closed arrows* and *triangles* indicate the major 3′ incision sites and the *open arrows* and *triangles* indicate the uncoupled 5′ incision site. The position of the substrate (141-mer) is also indicated.

ase results in complete inhibition of any nicking activity.

Order of Incisions—In *E. coli* (A)BC excinuclease the UvrB makes the 3′ incision and then UvrC makes the 5′ nick (Lin *et al.*, 1992). If UvrB fails to make the 3′ incision UvrC cannot incise 5′ even when it is bound to the UvrB·DNA complex (Lin and Sancar, 1992). The total lack of both 3′ and 5′ nicking activity in XP-F and ERCC-1 mutants and the proportional inhibition of both nicking by α-ERCC1 but not by α-XPG combined with the observation of uncoupled 5′ incision in the presence of α-XPG (Figs. 5–8) suggested that the opposite may be the case in humans: 5′ incision must be made by XPF·ERCC1 before XPG can incise at the 3′ side. This model would exclude the production of 3′ uncoupled nicks. In fact, we have never observed the bands corresponding to the 3′ uncoupled nicks in the HeLa CFE system. However, in a highly defined system which we have recently established (Mu *et al.*, 1995), a band (72-mer) generated by the possible uncoupled 3′ incision was often detected. Fig. 9*A* shows that excision is accompanied by the appearance of a 72-nt-long fragment only when all 5 fractions were used (*lane 2*), indicating that the production of the 72-mer is specific to human excinuclease activity. To confirm the specificity of this nick the excision reaction was performed in the presence of α-XPG. Fig. 9*B* shows that the antibodies specifically inhibit the appearance of the 72-mer fragment in addition to excised fragments (23–27-mers) by dual incisions while simultaneously enhancing the intensity of bands in the 91–96-mer region. These data are consistent with α-XPG inhibiting 3′ incision (72-mer) and excision (23–27-mers) without significantly affecting the 5′ incision. Thus, we conclude that the 72-mer band arose from uncoupled 3′ incision and that in humans 3′ and 5′ incisions can occur independent of one another under certain conditions.

DISCUSSION

Nucleotide excision repair, in general, is similar in *E. coli* and humans: a multisubunit-ATP dependent enzyme system removes damage by a dual incision mechanism. However, there are no sequence homologies between the proteins involved in the two systems. Furthermore, whereas at least 16 polypeptides are required for dual incisions in humans (Mu *et al.*, 1995), in *E. coli* 3 subunits are necessary and sufficient for dual incisions (Grossman and Thiagalingam, 1993; Sancar, 1994). In contrast to the differences between *E. coli* and humans, it appears that the excision repair systems in *Saccharomyces cerevisiae* and humans are very similar in all aspects including number and sequences of proteins involved (Prakash *et al.*, 1993; Friedberg *et al.*, 1995). Hence the discovery that yeast RAD2 protein (XPG homolog) had single-strand specific endonuclease activity (Habraken *et al.*, 1993) and also acted as a

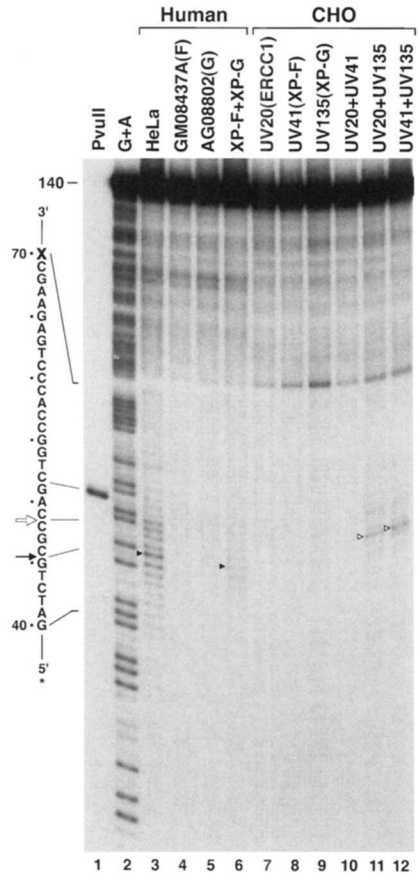

FIG. 7. **Incision assay with 5′-labeled substrate (5′ incision assay).** Fifty μg of HeLa CFE (*lane 3*) or mutant CFEs (*lanes 4, 5*, and *7–9*) were incubated with 5′ end-labeled substrate at 23 °C (to reduce nonspecific degradation) for 60 min. In complementation reactions (*lanes 6* and *10–12*), 25 μg from each cell line was premixed and then added to the reaction mixture. The products were resolved by electrophoresis on a 10% polyacrylamide sequencing gel. The products of *Pvu*II restriction digestion (*lane 1*) and the Maxam-Gilbert reaction of G+A (*lane 2*) were used for determining the incision sites. The *closed arrow* and *triangles* indicate the major 5′ incision site in human CFE and the *open arrow* and *triangles* indicate that in Chinese hamster ovary (*CHO*) CFE. The position of the substrate (140-mer) is also indicated. As determined by the intensity of the bands relative to the total amount of the DNA, 9.8% of the total radioactivity was detected in the 44–49-nt region with HeLa CFE (*lane 3*), in contrast to 1.6–2.2% (background) with mutant CFEs (*lanes 4, 5*, and *7–9*). In complementation reactions (*lanes 6, 11*, and *12*), 3.7–5.1% of that was detected in this region (*i.e.* approximately 37–52% complementation).

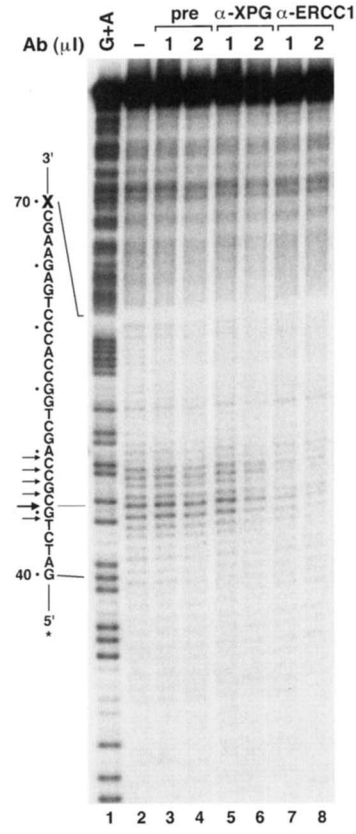

FIG. 8. **Effects of anti-XPG and anti-ERCC1 antibodies on the 5′ incision.** HeLa CFE (50 μg) was preincubated (for 10 min on ice) with the indicated amounts of antibodies (*Ab*), preimmune (*lanes 3* and *4*), α-XPG (*lanes 5* and *6*), or α-ERCC1 (*lanes 7* and *8*), and then added to the reaction mixture containing 5′ end-labeled substrate (23 °C, 60 min). The products were resolved by electrophoresis on a 10% polyacrylamide sequencing gel. *Lane 1* shows the Maxam-Gilbert reaction of G+A. The *arrows* indicate the 5′ incision sites.

5′→3′ exonuclease (Harrington and Lieber, 1994; Habraken *et al.*, 1994c) is relevant to the human system. Indeed, XPG has both activities (O'Donovan *et al.*, 1994a; Habraken *et al.*, 1994a, 1994c). Similarly, the XPF·ERCC1 complex which is the putative homolog of the RAD1·RAD10 endonuclease is expected to be an endonuclease (Tomkinson *et al.*, 1993; Sung *et al.*, 1993).

Since in *E. coli* UvrB has the 3′ incision activity and UvrC makes the 5′ incision (Lin and Sancar, 1992; Lin *et al.*, 1992), the discovery of endonuclease activities in RAD2 and RAD1·RAD10 led to the expectation that each of these nucleases made one of the incisions. Recent work with model substrates has led to specific predictions. Thus it was found that a truncated RAD2 incised a DNA with a single-stranded flap only when the flap had a 5′ terminus, leading to the prediction that RAD2 and therefore XPG must make the 3′ incision (Harrington and Lieber, 1994). A study with Y-shaped DNA and recombinant XPG protein also found that only the strand with 5′ single-stranded terminus was nicked and furthermore, that a DNA with a single-stranded bubble was nicked at the 3′ end of the bubble in the particular strand analyzed (O'Donovan *et al.*, 1994b) leading to the conclusion that XPG must make the 3′ incision. On the other hand, studies with RAD1·RAD10 complex and forked DNA showed that the strand with 3′ single-stranded terminus was nicked at the junction with the double-stranded region (Bardwell *et al.*, 1994) and hence it was suggested that RAD1·RAD10 (and XPF·ERCC1) must make the 5′ incision. However, an alternative model was advanced based on the finding that both XPG and RAD2 have intrinsic 5′→3′ exonuclease activity; it was, therefore, suggested that this finding was consistent with XPG making the 5′ incision and then partially degrading the excised oligomer to enable helicases to displace the excision product (Habraken *et al.*, 1994c). Two other observations are consistent with this model. First, the excision gap is not enlarged from the 3′ incision site (Huang *et al.*, 1992); second, the excised oligomer is degraded in 5′→3′ direction in CFE (Svoboda *et al.*, 1993). Hence, even though studies with model systems have been highly informative, they are not conclusive. Experiments with damaged substrates are needed to assign the roles of XPG and XPF·ERCC1 in the two incision reactions. In the present study, we used the excision and incision assays with damaged DNA and specific antibodies for the incision enzymes, and demonstrated that XPG makes a major incision at the 3rd phosphodiester bond **3′** to the lesion and XPF·ERCC1 complex incises mainly at the 25th phosphodiester bond **5′** to the lesion.

Two reports in the literature deal with substrates containing DNA lesions and CFEs from mutant cell lines to analyze roles of the subunits on incision reaction. In one, it was shown that

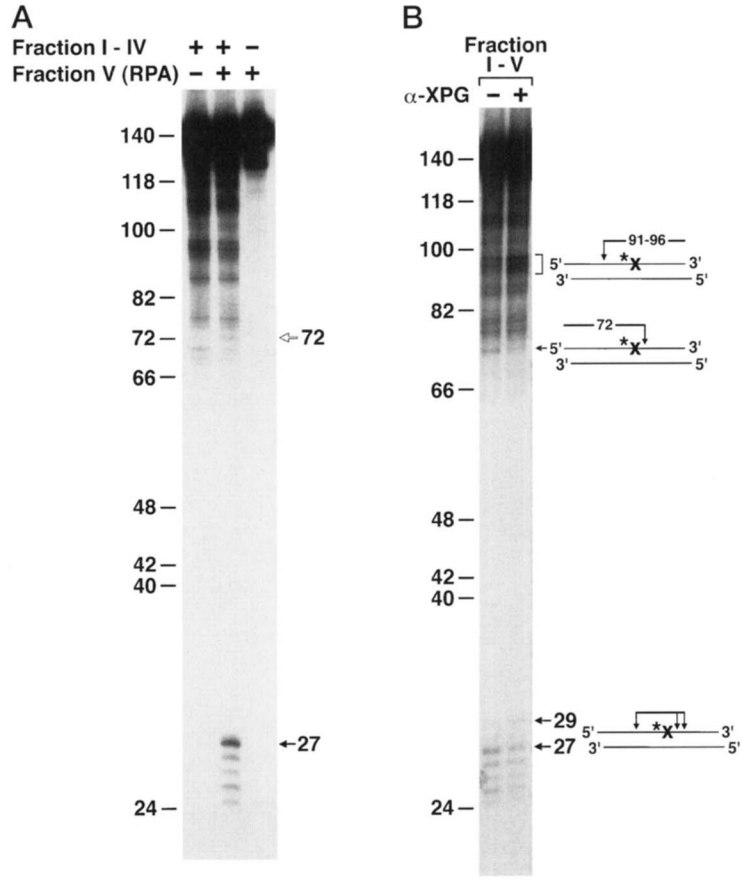

FIG. 9. **Effects of anti-XPG antibodies on the reconstituted excision repair system.** *A*: *lane 1*, Fractions I-IV; *lane 2*, Fractions I-V; *lane 3*, Fraction V (replication protein A, RPA) alone. The excision reaction with the internally labeled substrate (140-bp) was carried out as described under "Experimental Procedures" except that dNTPs were omitted. The products were resolved by electrophoresis on a 10% polyacrylamide sequencing gel. The positions of DNA size markers (ϕX174/*Hin*fI and ϕX174/*Hae*III) are indicated on the left margin. The *closed* and *open arrows* show the major excision product (27-mer) and the novel band (72-mer) observed in *lane 2*, respectively. *B*, α-XPG (1 μl) was added to the mixture of Fractions I-V and preincubated (for 10 min on ice). The analysis of excision products was carried out as described in *A*. The positions of DNA size markers (ϕX174/*Hin*fI) and the excision products (27- and 29-mers) are indicated. *Right panel*, schematic drawings of the incision pattern of human excinuclease on the internally labeled 140-bp substrate.

CFEs from XP-C, -D, and -G incised weakly at the 3rd phosphodiester bond 3′ to a single thymine dimer (Tateishi *et al.*, 1993), leading one to suspect that perhaps these subunits were not needed for 3′ incision. The other report presented data showing an incision by HeLa CFE at about 420 nt from the 3′ end of a fragment which contained a GTG-cisplatin adduct at position 424. It was also stated that extract from an ERCC-1 mutant cell line (43–3B) incised weakly at this position whereas CFE from an XP-G cell line showed little or no damage-dependent incision at this position (O'Donovan *et al.*, 1994b), suggesting that XPG but not ERCC1 was needed for 3′ incision. These contradictory results can be reconciled by assuming that the "incisions" detected in these types of experiments may sometimes be the products of nonspecific 3′→5′ or 5′→3′ exonucleases which degrade DNA starting from an end or a nonspecific nick in the substrate. Most such nucleases are blocked by lesions either at the lesion site or 1 to 2 nucleotides prior to the lesion giving the appearance of a specific nick. Indeed such an effect is seen in our Figs. 5 and 7. This phenomenon is not unique to a particular cell line; it shows considerable variability between extracts from the same cell line. In this paper, we show that the incisions are specific to excinuclease activity based on the complementation experiments and specific inhibition by the relevant antibodies.

The second mechanistically significant finding of our work deals with the order of incision. In *E. coli* if UvrB (because of active site mutation) is unable to make the 3′ incision, UvrC fails to make the 5′ incision. On the other hand if active-site mutant UvrB is loaded onto damaged DNA containing the 3′ incision, addition of UvrC to the UvrB-preincised DNA complex leads to quantitative 5′ incision and hence excision. In contrast, when active site mutant UvrC binds to UvrB·DNA complex, UvrB does make (uncoupled) 3′ incision quantitatively. Thus, it was concluded that in UvrB·UvrC·DNA complex production of 3′ incision by UvrB leads to a conformational change in the complex enabling UvrC to make the 5′ incision (Lin *et al.*, 1992). The inhibition of both 3′ and 5′ incisions by α-ERCC1 and the detectability of uncoupled 5′ incision in our experiments led us to suspect that in human excinuclease also there was an order of incision with 5′ preceding the 3′ incision. However, further experiments with the defined system revealed the presence of uncoupled 3′ incision (Fig. 9). Thus it is possible that in humans as well 3′ incision precedes 5′ incision, but that in the presence of α-XPG the enzyme system is induced to make 5′ incision only. Whether uncoupled 5′ incision occurs at all in the absence of antibodies remains to be determined.

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