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Rodent UV-sensitive mutant cell lines in complementation groups 6–10 have normal general excision repair activity

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ABSTRACT

Mammalian nucleotide excision repair is the primary enzymatic pathway for removing bulky lesions from DNA. The repair reaction involves three main steps: (i) dual incisions on both sides of the lesion; (ii) excision of the damaged base in an oligonucleotide 24–31 nt in length; (iii) filling in of the post-excision gap and ligation. We have developed assays that probe the individual steps of the reaction. Using these methods (assays for incision, excision and repair patch synthesis), we demonstrate that the mammalian excision nuclease system removes bulky lesions by incising mainly at the 22nd–25th phosphodiester bonds 5′ and the 3rd–5th phosphodiester bonds 3′ of the lesion, thus releasing oligonucleotides primarily 26–29 nt in length. The resulting excision gap is filled in by DNA polymerases δ and ε as revealed by the 'phosphorothioate repair patch assay'. When these assays were employed with cell-free extracts from the moderately UV-sensitive rodent mutants in complementation groups 6–10, we found that these mutants are essentially normal in all three steps of the repair reaction. This leads us to conclude that these cell lines have normal *in vitro* repair activities and that the defects in these mutants are most likely in genes controlling cellular functions not directly involved in general excision repair.

INTRODUCTION

Nucleotide excision repair (excision repair) is the primary mechanism for removing DNA damage caused by UV light and by agents which make bulky base adducts (1–3). Lack of excision repair causes xeroderma pigmentosum (4,5), which may result from mutations in seven genes (XPA, XPB, XPC, XPD, XPE, XPF and XPG). In addition, mutations in two genes, CSA and CSB, abolish transcription-coupled repair and cause Cockayne's syndrome (6,7). To supplement these naturally occurring human mutations, rodent cell lines sensitive to UV and UV mimetic agents have been isolated and characterized. These fall into 11 complementation groups, some of which are the same as those groups defined by XP and CS mutations: group 2 (XPD), group 3 (XPB), groups 4 and 11 (XPF), group 5 (XPG), group 6 (CSB) and group 8 (CSA) (8). It has also been shown that the protein defective in group 1, ERCC1, is in a complex with XPF (9,10), which constitutes the 5′ nuclease (11) of the human dual incision–excision nuclease system (12).

The excision repair nuclease has recently been reconstituted with proteins purified to near homogeneity (13). This study revealed that six repair factors consisting of XPA, RPA, TFIIH (XPB, XPD, p62, p44, p34), XPC, XPF–ERCC1 and XPG are necessary and sufficient for the initial steps of excision repair resulting in high efficiency damage removal. Similar results have been obtained with the structurally and functionally homologous *Saccharomyces cerevisiae* proteins (14). These studies raised questions as to the direct participation in general excision repair of the proteins defective in rodent complementation groups 7, 9 and 10. The present study was undertaken to directly address these questions, as well as to determine the possible effects of the CSA and CSB proteins (groups 8 and 6) on basal excision repair.

Since excision repair encompasses dual incisions, excision of 24–31 nt oligomers and repair synthesis to fill in the excision gap, cell lines representative of groups 6–10 were tested for each of these functions. Cell-free extracts were tested for 5′ incision (both the location and level of activity), for the level of excision as well as the size(s) of the excision products and for the precise boundaries of the repair synthesis patch using the high resolution phosphorothioate repair patch assay (15). Our results demonstrate excellent correlation between the size of the excision product and the repair patch and show that representatives of complementation groups 6–10 incise at the normal sites, excise damage at a normal rate and generate a resynthesis patch indistinguishable from the wild-type repair patch. We conclude that these mutants are not defective in the basal steps of general excision repair.

MATERIALS AND METHODS

Materials

The oligodeoxyribonucleotides used for substrate preparation were from either Operon Technologies (Alameda, CA) or

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Midland Certified Reagent Co. (Midland, TX). A 20mer containing a cyclobutane thymine dimer (T<>T) was a kind gift from J.-S.Taylor (Washington University, St Louis, MO). T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) and [γ-^{32}P]ATP was from ICN (Irvine, CA). Nucleotide triphosphates (dNTP) were from Pharmacia (Piscataway, NJ) and nucleotide thiotriphosphates (dNTPαS) were from Amersham Life Science (Arlington Heights, IL).

Cell lines and preparation of cell-free extracts

Repair-proficient HeLa and Chinese hamster ovary (CHO) cell lines AA8 and K1 and the UV-sensitive mutant derivatives listed below were used in this study: UV41 (group 4), UV135 (group 5), UV61 (group 6), V-B11 (group 7), US31 (group 8), CHO7PV (group 9), CHO4PV (group 10) and UVS1 (group 11). Cells grown either as a suspension or adherent culture in Eagle's MEM supplemented with 10% fetal bovine serum were harvested and cell-free extracts (CFE) prepared as described (16) and stored at –80°C in 25 mM HEPES, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol and 12.5% glycerol (v/v) buffer. The CFE concentrations were 10–25 mg/ml and the extracts were stable for at least two cycles of thawing and refreezing.

Substrates

Substrates containing either a centrally located cholesterol residue (in place of T at position 70) or a T<>T adduct at nt 74–75 were prepared as described (17) by phosphorylating with T4 polynucleotide kinase, annealing and ligation of six overlapping oligonucleotides to generate a 140 bp duplex. Four types of substrates were used: 5′-end-labeled DNA, either with no damage or with the cholesterol lesion, was used for the incision and resynthesis (repair patch) assays and internally labeled DNA, with either the cholesterol residue or the T<>T photoproduct, was used for the excision assay.

Incision assay

This assay measures cleavage 5′ of the lesion using 5′-end-labeled DNA as the substrate. The reaction mixtures contained 3–15 fmol radiolabeled DNA with a cholesterol residue, 50 µg CFE and 12.6 fmol pBR322 in 25 µl reaction buffer (35 mM HEPES, pH 7.9, 10 mM Tris pH 7.5, 60 mM KCl, 40 mM NaCl, 5.6 mM $MgCl_2$, 0.4 mM EDTA, 0.8 mM dithiothreitol, 2 mM ATP and 3.2% glycerol with bovine serum albumin at 0.2 mg/ml). The reaction was carried out at 25°C for 60 min or at 30°C for 45 min. DNA was deproteinized with proteinase K (0.2 mg/ml for 15 min at 37°C), followed by phenol, phenol/chloroform and ether extractions and then precipitated with ethanol in the presence of 20 µg oyster glycogen. Recovered DNA was resuspended in formamide/dye mixture and resolved on 12% denaturing polyacrylamide gels. Following autoradiography to visualize the repair products, the level of 5′ incision (oligomers in the 42–53 nt range) was quantified by scanning the dried gel with an AMBIS systems scanner.

Excision assay

Using internally labeled DNA substrate (at the 6th phosphodiester bond 5′ of the cholesterol residue or at the 13th bond 5′ of T<>T), this assay detects the excised fragment resulting from both 5′ and 3′ incisions. The 25 µl repair reaction was supplemented with 20 µM each of dATP, dCTP, dGTP and TTP. The reaction was initiated by addition of substrate DNA (2–6 fmol cholesterol-containing 140mer or 16 fmol T<>T substrate) and 50 µg CFE and was incubated at 30°C for 45–60 min. Following resolution on 10% sequencing gels and autoradiography to visualize the repair products, the level of excision (oligomers in the 24–31 nt range) was quantified by scanning dried gels with an AMBIS systems scanner.

Repair synthesis (patch) assay

This assay measures the repair patch at single nucleotide resolution. Terminally labeled DNA (10–20 fmol) was incubated with 40–70 µg CFE in 25 µl reaction buffer containing 80 µM each of three dNTPs and 80 µM of one dNTPαS and the reaction was at 30°C for 90 min. To obtain the precise patch sequence with wild-type CFE, four separate reactions were carried out with a different dNTPαS in each reaction. Only the dGTPαS- and TTPαS-containing reactions were performed for analysis of repair patches made by mutant CFEs. Following the repair reaction, deproteinized DNA was resolved on 8% denaturing polyacrylamide gels and full-length (140 nt) DNA was located by autoradiography and recovered by electroelution. Following ethanol precipitation of the eluted DNA, samples were resuspended in 9 µl 20 mM HEPES, pH 7.5, heated for 3 min at 70°C and cooled to 25°C. Iodine was added to 0.5 mM and the mixture was incubated at 25°C for 1 min (18). The reaction was quenched by addition of sodium acetate, pH 5.2, to 0.25 M and DNA was precipitated with ethanol, resuspended in formamide/dye mixture and resolved on 12% sequencing gels without heating prior to loading onto the gel. The repair patch was visualized by autoradiography.

RESULTS

Incision, excision and repair patch assays

We wished to examine the repair activity of mutant cell lines in the moderately UV-sensitive complementation groups 6–11, whose representative cell lines are less sensitive than the original mutants belonging to complementation groups 1–5 (8,19,20). To this end we employed assays that probe the three steps of basal excision repair: incision, excision and repair synthesis and ligation. Although the incision and excision assays have been used in earlier studies of excision repair with CFEs (9,17), the high resolution repair patch assay has not previously been used in a mammalian *in vitro* system.

Each of the repair assays was performed with normal, repair-proficient CFEs (HeLa, CHO K1 or CHO AA8) prior to use with mutant CFEs. All three cell lines gave similar results and the data obtained with the CHO K1 extract are shown in Figure 1. The incision assay, which utilizes a 5′-end-labeled substrate, detects incision sites 5′ of the lesion and also 3′ incision sites when this incision is not accompanied by the 5′ incision (3′ uncoupled incision). The CHO K1 CFE primarily incises at the 22nd–25th bonds 5′ of the lesion (Fig. 1); this agrees with an earlier study using this substrate and HeLa CFE which generates a strong

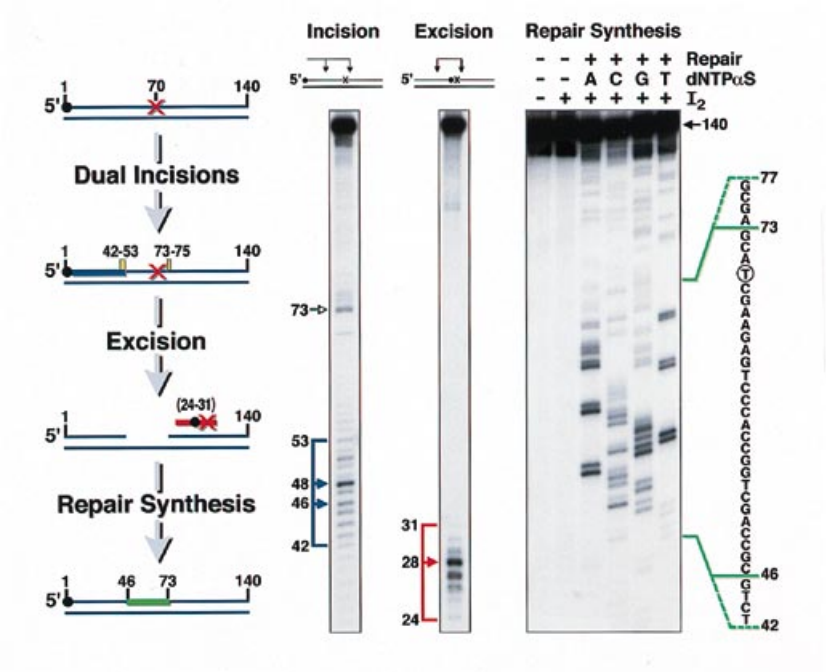

Figure 1. Excision repair assays. The schematic drawing on the left shows the three basic steps of nucleotide excision repair. A 140 bp duplex with a cholesterol substituent at position 70 was the substrate. In the incision assay the repair reactions produce radiolabeled fragments extending from the 5′-end to the incision sites (blue). In the excision assay the repair reaction produces radiolabeled fragments 24–31 nt in length (red). In the repair synthesis assay 5′-terminally labeled DNA is used as substrate together with dNTPαS and the resulting repair patch (green) can be visualized by partial hydrolysis with iodine. The autoradiographs show the actual data obtained with CHO K1 CFE. In the incision assay, the primary 5′ incision products are in the 42–53 nt range (blue bracket); the 73mer generated primarily by non-specific nucleases is indicated with an open arrow. In the excision assay, the excision products are in the 24–31 nt range (red bracket), with the prominent 28mer indicated by an arrowhead. The repair synthesis assay reveals a prominent repair patch indicated by a green bracketed area (nt 46–73), while dotted lines indicate the less pronounced regions of resynthesis which extend 4 nt in either direction; the sequence of the repair patch is shown to the right of this panel with the circled T indicating the position of the lesion that was removed.

incision site the 25th phosphodiester bond 5′ of the cholesterol residue and weaker incisions at the 22nd–24th phosphodiester bonds (17). Also consistent with earlier work, the range of 5′ incision sites covers about a full turn of the helix (13,17). At a low frequency, some 3′ uncoupled incision may be observed at the 3rd phosphodiester bond 3′ of the cholesterol residue; however, since a band at this position is also observed with mutant extracts known to be defective in incision (Fig. 2, lane 4, and data not shown), this band cannot definitively be ascribed to uncoupled 3′ incision. This 73 nt fragment, which is particularly strong in rodent cell lines compared with human lines, is most likely caused by both 3′ uncoupled incisions and a strong, non-specific 3′→5′ exonuclease which is blocked by the cholesterol residue (17). When 5′-end-labeled, unmodified DNA was used for the incision assay (data not shown), distinct incisions were not observed; instead we detected a DNA ladder, indicating low levels of nicking at random positions.

The excision assay utilizes an internally labeled substrate to monitor dual incisions both 5′ and 3′ of the damage. The cholesterol residue is removed primarily in oligomers 26–29 nt in length; in addition to these major excision products, other fragments in the range 24–31 nt are excised at a lower frequency (Fig. 1).

The repair patch assay, based on the phosphorothioate sequencing method developed by Gish and Eckstein (21) and adapted for precise measurement of repair patches by Sibghat-Ullah *et al.* (15), is quite informative. Briefly, terminally labeled substrate is used in a reaction mixture where three dNTPs plus one dNTPαS are used as the nucleotide source for gap filling following excision. Four reactions are carried out, each containing a different base in the dNTPαS form. Following repair synthesis, the DNA is treated with iodine, which preferentially cleaves phosphorothioate linkages (partial hydrolysis). When the four separate reactions are analyzed in parallel, the sequence and, hence, the precise boundaries of the repair patch are revealed. Figure 1 shows a striking 28 nt repair patch, which is in excellent agreement with the size of the predominant excision product and with the location of the 5′ incision sites. Thus, in mammalian cells, as in *Escherichia coli* (15), the excision gap is filled and ligated without extensive enlargement at the 5′ incision site or by nick translation from the 3′ incision site. It must be noted that the mammalian repair patch extends ~4 nt in either direction of the main patch. This is most likely a manifestation of the variable incision sites on both sides of the lesion (2,12); however, the possibility of low levels of gap enlargement during repair synthesis cannot be formally eliminated. When unmodified DNA was used for the repair patch assay (data not shown), we observed low levels of dNTPαS incorporation throughout the molecule, consistent with the random nicks observed in the 5′ incision assay.

Incision activity in complementation groups 6–10

The 5′ incision site was localized using CFE and 5′-end-labeled cholesterol substrate. With this system and HeLa extract, bands

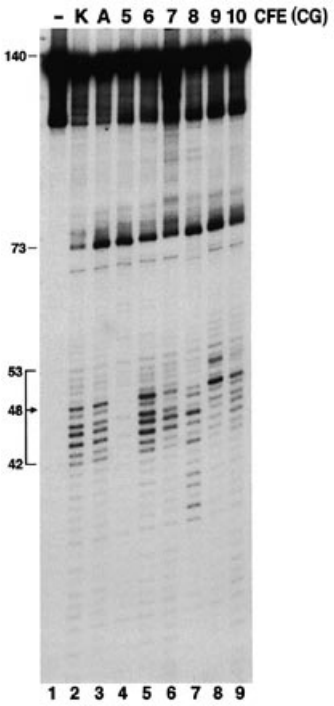

Figure 2. Incision activity of CFE from UV-sensitive rodent cell lines. Qualitative analysis of incision activity for complementation groups 5–10 (lanes 4–9) relative to parental cell lines CHO K1 (K, lane 2) and AA8 (A, lane 3); 5′-terminally labeled cholesterol substrate alone is shown in lane 1. The bracket indicates the major 5′ incision sites. The strong band at position 73 results from exonucleolytic degradation of substrate up to the vicinity of the cholesterol substituent.

corresponding to incisions at the 18th–29th phosphodiester bonds 5′ of the lesion are detected, with the major band being at the 25th phosphodiester bond (17). When the incision assay was conducted with CFEs from complementation groups 6–10, the mutant extracts gave incision patterns similar to those observed with wild-type CHO cell lines (Fig. 2), whereas there were no bands above background in CFEs from groups 1–5 (lane 4, and data not shown). With this assay the background caused by non-specific nucleases is relatively high, particularly with the group 8 CFE (lane 7), which results in an apparent shift of the specific incision bands to smaller fragments with the distal incision at nt 35 compared with nt 42 for other rodent extracts. Quantitative analysis of Figure 2 shows that 1.6% of the radioactivity is in the 42–53 nt region for the excision-defective group 5 (XPG) CFE and the values for the wild-types (lanes 2 and 3) are 5–6% compared with 5–8% for groups 6–10 (lanes 5–9). Thus, within the limitations of this assay, we conclude that complementation groups 6–10, with the possible exception of group 8, have normal 5′ incision activity.

Excision activity in complementation groups 6–10

CFEs from rodent complementation groups 5–11 were tested for excision activity using internally labeled substrates containing either a cholesterol residue or a T<>T photoproduct. Figure 3A shows that, with the exception of CFEs from groups 5 and 11, all tested extracts have similar excision activity with the cholesterol substrate. Complementation group 5 is known to be mutated in XPG (22) and group 11 has recently been found to be the equivalent of group 4, which is mutated in the XPF gene (10,23–25). Thus, it is not surprising that no or greatly reduced excision activity is observed with these extracts. Quantitative analyses of several excision assays, such as that shown in Figure 3A, are summarized in Figure 3B. With this substrate CFEs from groups 9 and 10 exhibited reduced levels of excision compared with groups 6–8, but clearly were in the range of normal excision activity as defined by the levels of excision observed with the wild-type cell lines K1 and AA8. We also conducted kinetic assays in which the extract concentrations ranged from 0.5 to 2.5 mg/ml or the times of incubation varied from 15 to 120 min (data not shown), but no clear differences were observed.

Although the level of excision with all extracts was ~10-fold lower when the T<>T substrate was used, similar levels of excision were observed for all tested CFEs (Fig. 4A and B). When the T<>T photoproduct is in the sequence context GCTCGAGC-TAAAT<>TCGTCAG, the primary excision products are of equal intensity and are 24–31 nt in length. Thus, we conclude that complementation groups 6–10 have normal *in vitro* excision activity with both the cholesterol and the T<>T substrate.

Repair patch of complementation groups 6–10

The data presented thus far are consistent with normal incision and excision activities for the representative cell lines of groups 6–10. We wished to know if these cell lines have a detectable defect in the repair synthesis and ligation steps of excision repair. For this purpose we conducted phosphorothioate repair patch assays (12,15) with the mutant CFEs using only two nucleotides in the dNTPαS form. The results are shown in Figure 5. A normal sequence pattern is observed for the wild-type CHO K1 (lanes 1 and 2) but no patch is seen with XPF/group 4 (lanes 3 and 4) or XPG/group 5 (lanes 5 and 6) extracts. The patches for groups 6–10 (lanes 7–16) are qualitatively similar to those observed for the wild-type, although group 8 exhibits a patch enlarged by ~4 nt at the 5′ boundary (lane 12), consistent with the observed shift in 5′ incision sites observed with this extract (see Fig. 2, lane 7). Thus, we conclude that complementation groups 6–10, with the possible exception of group 8, have normal repair synthesis and ligation activities.

DISCUSSION

Our data show that rodent UV-sensitive mutant cell lines in complementation groups 6–10 are normal in the incision, excision and resynthesis and ligation steps of nucleotide excision repair when assayed *in vitro*. These findings support the recent reconstitution experiments with purified proteins which concluded that XPA, RPA, XPC, TFIIH, XPF/ERCC1 and XPG were necessary and sufficient for a high efficiency excision reaction (13,26,27). This leads us to consider other explanations for the UV-sensitive phenotypes of complementation groups 6–10.

The prototypic representative of complementation group 6 is UV61 (28,29), which was reported to remove (6–4) photoproducts but not cyclobutane dimers (30). Cloning of the gene (*ERCC6*, *CSB*) and related analyses demonstrated that this cell line is deficient in transcription–repair coupling (31). Consistent with our previous data showing normal excision of T<>T from a semisynthetic covalently closed plasmid substrate (9), we here demonstrate that UV61 extracts have normal incision and

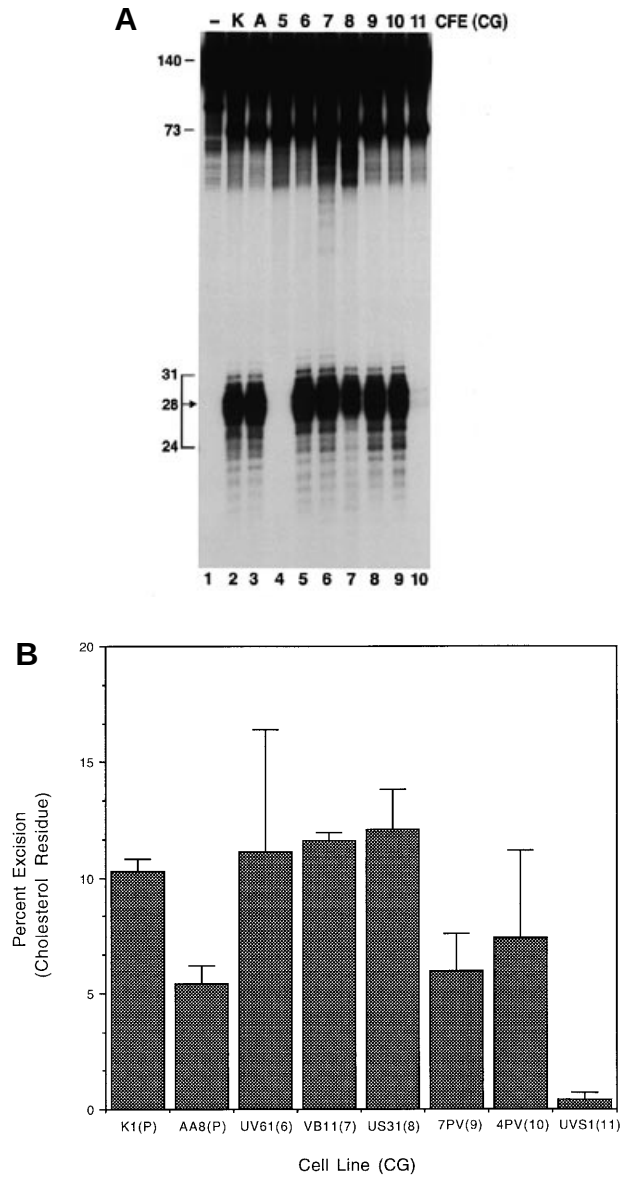

Figure 3. Excision activity of mutant cell lines with cholesterol-containing substrate. (**A**) Qualitative analysis of excision activity for complementation groups 5–11 (lanes 4–10) relative to parental cell lines CHO K1 (K, lane 2) and AA8 (A, lane 3); internally labeled cholesterol-containing substrate DNA alone is shown in lane 1 and the bracket indicates the major excision products. (**B**) Quantitative analysis of excision activity for complementation groups 6–11 relative to CHO K1 and AA8; the average of two independent experiments and the standard error are shown. Note that complementation group 11, which is known to be identical to group 4 (XPF), has ~3% residual activity relative to the other groups.

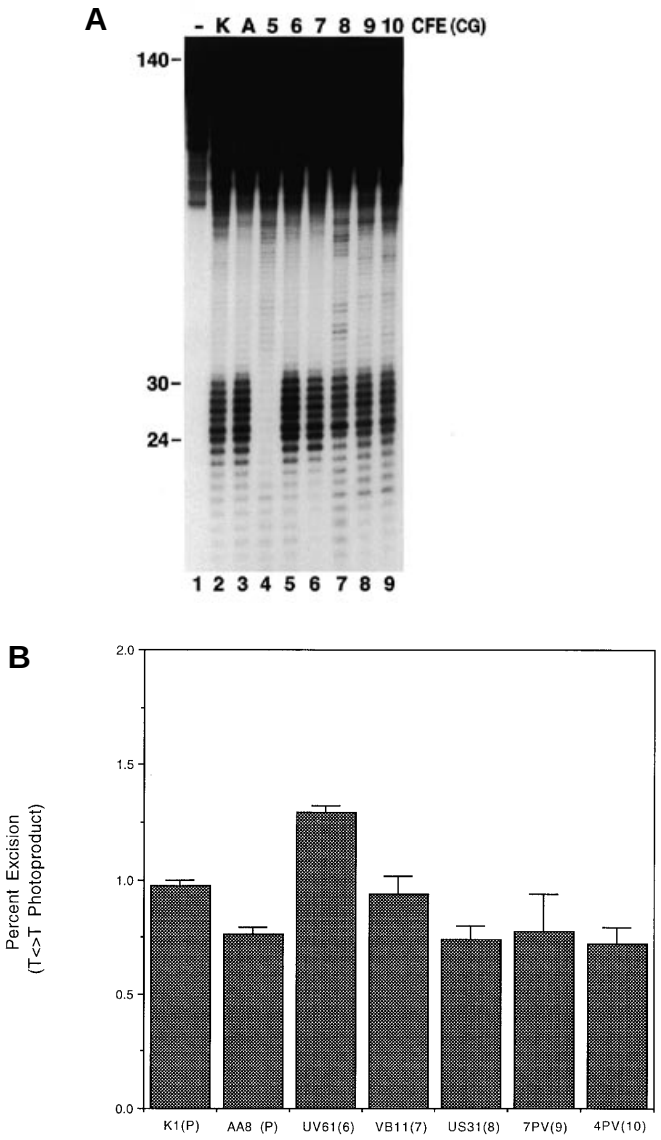

Figure 4. Excision of T<>T by mutant CFEs. (**A**) Qualitative analysis of excision activity for complementation groups 5–10 (lanes 4–9) relative to parental cell lines CHO K1 (K, lane 2) and AA8 (A, lane 3); internally labeled T<>T-containing substrate DNA alone is shown in lane 1. (**B**) Quantitative analysis of excision activity for complementation groups 6–10 relative to CHO K1 and AA8; the average of two independent experiments and the standard error are shown.

excision activities on another bulky lesion as well as on a T<>T photoproduct contained within a linear duplex molecule. Thus, our data support earlier conclusions that the apparent lack of excision of Pyr<>Pyr *in vivo* is due to a defect in strand-specific repair (6,31,32). Furthermore, the normal *in vitro* incision, excision and repair synthesis observed for UV61 extracts suggest that the CSB protein does not have an accessory role in the basal repair reaction.

The V-B11 cell line (complementation group 7) was isolated as a moderately UV-sensitive cell line (33) with normal unscheduled DNA synthesis (UDS) but a 70% reduction in incision activity. It was also reported that it removes (6–4) photoproducts at a normal rate from the genome overall, but excises T<>T from a transcriptionally active locus at 20% of the rate of the parental line (34). Superficially, these phenoytpic characteristics are similar to those of Cockayne's syndrome cells, but somatic hybrids of V-B11 with other rodent cell lines demonstrate that this cell line complements both groups 6 and 8 (35,36). Although a subtle defect in transcription–repair coupling cannot be eliminated, our *in vitro* results of normal incision, excision and repair synthesis do not reveal any defect in basal/general excision repair in this mutant. Clearly, more work is needed to explain the UV sensitivity of this cell line.

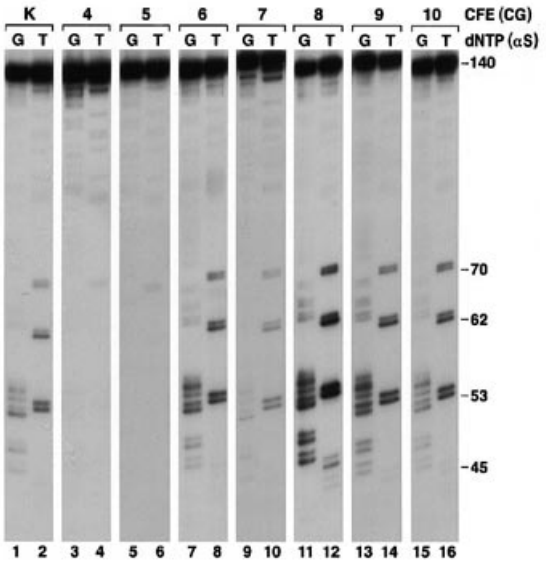

Figure 5. Resynthesis activity of mutant cell lines relative to a parental cell line. Qualitative analysis of resynthesis activity for complementation groups 4–10 (lanes 3–16) relative to parental cell line CHO K1 (K, lanes 1–2); repaired DNA was treated with I_2 as described in the text and the reaction products were resolved on 12% sequencing gels and visualized by autoradiography. The DNA fragments identified to the right are substrate DNA (140), the three thymidines in the patch (positions 70, 62 and 53) and the guanidine near the 5′ patch boundary (position 45).

The sole representative of complementation group 8 is the UV-sensitive mouse lymphoma cell line US31 (36,37). This cell line is also moderately UV sensitive and removes (6–4) photoproducts, but not Pyr<>Pyr, at a normal rate *in vivo*. Recently it was demonstrated that a derivative of this mutant cell line did not complement a human CSA^- cell line and that the cloned *CSA* gene restored UV survival to normal rates (38). CSA mutants, like CSB (group 6) mutants, are defective in transcription-coupled repair and our observations of normal excision and repair synthesis activities with a linear substrate are consistent with this phenotype. In our incision and repair patch assays we observed a 4–6 nt shift at the 5′ incision site with this mutant and it is conceivable that this may be a direct effect of the lack of CSA protein in the mutant CFE. However, the CFE from this cell line contains a potent non-specific nuclease which degrades input DNA (see lanes 7 of Figs 2, 3A and 4A). Hence, we consider this mutant to be normal in all aspects of basal excision repair. This conclusion is consistent with the finding that in a reconstituted excision nuclease system devoid of CSA the 5′ incision site corresponds to the 5′ incision site we detect with all other cell lines (13).

The sole members of complementation groups 9 and 10, CHO7PV and CHO4PV respectively, were isolated from the CHO K1 cell line and characterized as mutants with reduced UDS after UV irradiation (39). Detailed analyses revealed a 2- to 3-fold increased UV sensitivity for both cell lines with reduced excision of both (6–4) and Pyr<>Pyr photoproducts. With our *in vitro* assays we observe excision of the cholesterol residue and the T<>T photoproduct within the normal range.

The high efficiency reconstitution of the human excision nuclease with six well-defined factors (13) makes it unlikely that other proteins contribute to the efficiency of excision repair in our model system. We considered the possibility that groups 7, 9 and 10 might be leaky alleles of human repair proteins not previously correlated with rodent mutations (e.g. XPA, XPC-HHR23B or TFIIH subunits). To test these possibilities, mutant CFEs were supplemented with purified XPA, XPC-HHR23B or TFIIH (p89/XPD, p80/XPB, p62, p44 and p34) and tested for excision of the T<>T photolesion; no effect on excision activity was observed (data not shown). These results suggest, but do not definitively confirm, the possibility that groups 7, 9 and 10 are not leaky mutants of known excision repair genes.

Since our *in vitro* assays, which do not reveal repair defects in these cell lines, were conducted with naked DNA and not chromatin, it is quite possible that the proteins defective in groups 7, 9 and 10 are responsible for the enhanced accessibility of DNA (i.e. chromatin factors) to the other repair factors known to have an essential role in excision repair. Cloning these genes and characterization of the encoded proteins is likely to provide useful information on the repair of lesions in chromatin.

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