Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours

Beate Köberle*†, John R.W. Masters†, John A. Hartley‡ and Richard D. Wood*

Metastatic cancer in adults usually has a fatal outcome. In contrast, advanced testicular germ cell tumours are cured in over 80% of patients using cisplatin-based combination chemotherapy [1]. An understanding of why these cells are sensitive to chemotherapeutic drugs is likely to have implications for the treatment of other types of cancer. Earlier measurements indicate that testis tumour cells are hypersensitive to cisplatin and have a low capacity to remove cisplatin-induced DNA damage from the genome [2,3]. We have investigated the nucleotide excision repair (NER) capacity of extracts from the well-defined 833K and GCT27 human testis tumour cell lines. Both had a reduced ability to carry out the incision steps of NER in comparison with extracts from known repair-proficient cells. Immunoblotting revealed that the testis tumour cells had normal amounts of most NER proteins, but low levels of the xeroderma pigmentosum group A protein (XPA) and the ERCC1–XPF endonuclease complex. Addition of XPA specifically conferred full NER capacity on the testis tumour extracts. These results show that a low XPA level in the testis tumour cell lines is sufficient to explain their poor ability to remove cisplatin adducts from DNA and might be a major reason for the high cisplatin sensitivity of testis tumours. Targeted inhibition of XPA could sensitise other types of cells and tumours to cisplatin and broaden the usefulness of this chemotherapeutic agent.

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Results and discussion

Low levels of NER by extracts from testis tumour cell lines

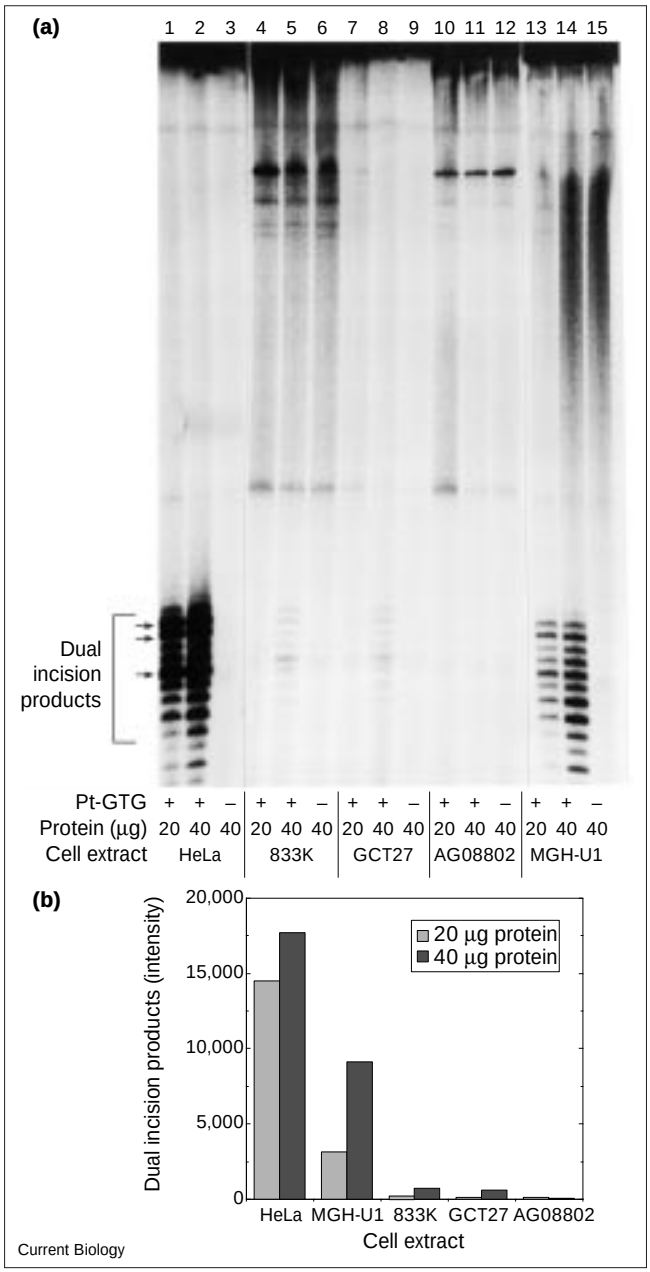
The only known mechanism by which cisplatin lesions are removed from DNA in mammalian cells is by NER. This involves steps of damage recognition, opening of DNA, and incision on both sides of a lesion. The core reaction requires the proteins XPA, RPA, XPC–hHR23B, TFIIH, ERCC1–XPF and XPG [4,5]. To analyse the NER capacity of cisplatin-hypersensitive human testis tumour cell lines, we measured the ability of cell extracts to form dual incisions flanking an adduct. A plasmid was used containing a single 1,3-intrastrand d(GpTpG) cisplatin cross-link (Pt-GTG), which is repaired efficiently by the mammalian NER system [6]. The 24–32 nucleotide products produced by dual incision are detected by end-labelling with the aid of a complementary oligonucleotide [7], separated on a denaturing polyacrylamide gel and quantified.

Incubation with HeLa cell extract produced the characteristic pattern of dual incision products (Figure 1a, lanes 1,2). No incision was detected with a non-adducted plasmid (Figure 1a, lane 3). Similarly, cell extracts from the human bladder tumour line MGH-U1 were proficient in NER, showing about 50% of the activity of HeLa extract when 40 μg protein was used (Figure 1a, lanes 13–15). In contrast, 40 μg protein from extracts of the testis tumour cell lines 833K and GCT27 gave a weak pattern of dual incision (Figure 1a, lanes 5,8), with products at about 10% of the level obtained with MGH-U1 or with extracts from other repair-proficient cell lines. Extracts from the completely XPG-defective cell line AG08802 yielded no dual incision products (Figure 1a, lanes 10,11). All findings were reproduced in additional experiments using at least four independent extracts from each tumour cell line. The results show that the testis tumour cell lines have unusually low constitutive levels of NER. This is consistent with observations that these cells have a low capacity to remove cisplatin adducts [2,3].

Reduced amounts of XPA protein and ERCC1–XPF complex in the testis tumour cells

Our hypothesis was that the low NER capacity of the testis tumour cells might be caused by a reduced level of one or more core repair factors. Levels of NER proteins were measured by immunoblotting a panel of cells including the two testis tumour cell lines 833K and GCT27, the bladder tumour line MGH-U1, HeLa cells and a normal lymphoblastoid cell line 705ori. In testis tumour cell lines, most of the repair proteins were present at levels equivalent to or even greater than the other cell lines, as shown for the RPA p34 subunit (Figure 2a), XPG and subunits of TFIIH (Figure 2b) and XPC protein (Figure 2c). The RPA and TFIIH subunits can be regarded as internal controls for the amount of nuclear protein added, as they are housekeeping proteins necessary for DNA replication and

Figure 1



(a) Autoradiograph showing repair of a cisplatin adduct after incubation with extract protein (20 or 40 μg at 2 or 4 mg/ml) from HeLa cells, the bladder cancer cell line MGH-U1, the testis tumour lines 833K and GCT27 and the NER-defective XP group G AG08802 cell line. Reaction mixtures contained either 50 ng of a duplex plasmid with the Pt-GTG adduct (+) or 50 ng control DNA synthesised without the adduct (–). Dual incision products were detected by direct labelling as described [7] and are indicated by the bracket on the left. Arrows point to the bands arising from cleavage at the three most frequent 5′ incision sites in HeLa cells to yield fragments 26, 29 and 30 nt long. (b) The data from (a) quantified by measuring the labelled dual incision products.

transcription, respectively. Two factors were consistently found in reduced amounts in testis tumour cells. The amount of XPA protein was reduced to 25% or less in 833K and GCT27 compared with MGH-U1, HeLa and 705ori cells (Figure 2d). In addition, the amounts of ERCC1–XPF factor were reduced in 833K and GCT27 cells compared with the other three cell lines (Figure 2e).

Restoration of testis tumour cell extract NER with purified XPA protein

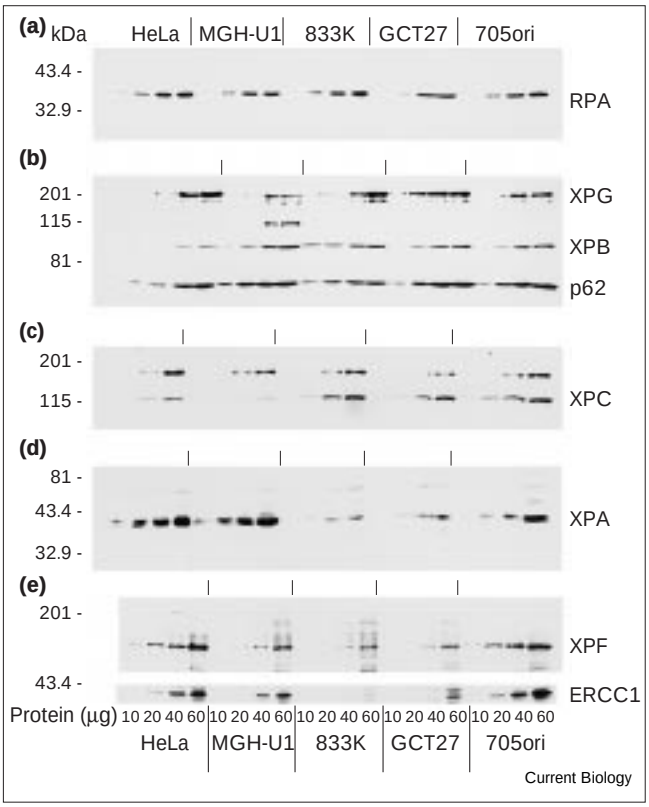
To determine whether the NER deficiency of the testis tumour cell extracts could be restored, extracts were supplemented with the proteins that were present in low amounts, XPA and ERCC1–XPF. Addition of 10 ng XPA markedly stimulated the dual incision activity of the testis tumour cell extracts (Figure 3a, lanes 7,8 and lanes 13,14), with little effect on the bladder tumour MGH-U1 cell extract (lanes 1,2). Quantification showed an approximately fourfold stimulation by 10 ng XPA in the testis tumour cell extracts. As a control, XPA protein conferred repair capacity on completely NER-defective XP group A extract (lanes 19,20). Addition of ERCC1–XPF complex also stimulated repair by extracts from the cell lines, but the effect was less marked. Supplementation with purified ERCC1–XPF resulted in twofold stimulation of 833K extract repair activity (Figure 3b, lanes 5–8), as well as a nearly twofold stimulation of MGH-U1 extract repair (Figure 3b, lanes 1–4). As a control, ERCC1–XPF fully restored repair to ERCC1-defective control cell extracts (Figure 3b, lanes 9–12). ERCC1–XPF had a modest effect on GCT27 cell extract (Figure 3c, compare lanes 3,5). Quantitative immunoblotting, using purified proteins as standards, showed that 40 μg MGH-U1 protein extract contained about 10 ng of XPA and about 20 ng of ERCC1–XPF and so the amounts of proteins added in Figure 3 are in the physiological range.

Experiments with combinations of factors showed that XPA had by far the most pronounced effect on repair by the testis tumour cell extracts. For example, addition of 40 ng XPA protein increased the dual incision activity of GCT27 extract by 6-fold, with a 1.8-fold further enhancement given by combination with ERCC1–XPF complex (Figure 3c, compare lanes 3,4,6). The reaction was specific and repair was not enhanced significantly by other NER proteins. Addition of XPG protein did not influence the dual incision activity of GCT27 or 833K extracts (Figure 3c, compare lanes 3,7 and 9,13). Similarly, addition of XPC or XPC–hHR23B complex did not stimulate repair by 833K and GCT27 extracts (data not shown).

Implications for tumour sensitivity and chemotherapy

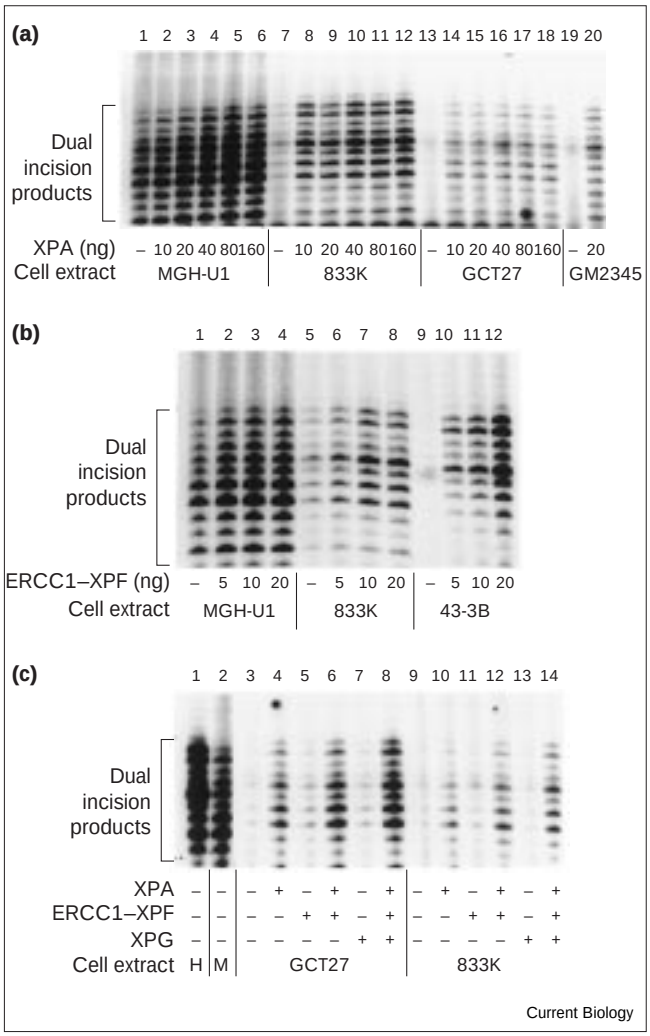
These results show that the low amount of XPA protein in the testis tumour cell lines is a sufficient explanation for their low NER capacity and indicate that this is a major factor determining their drug sensitivity. Consistent with this, XPA-defective cells are hypersensitive to killing by cisplatin–DNA lesions [8,9]. The XPA protein is at the

Figure 2



NER protein subunits in extracts from HeLa, MGH-U1, 833K, GCT27 and 705ori cell lines. For each cell line 10, 20, 40 and 60 μg protein extract were separated by SDS–PAGE and immunoblotted for **(a)** RPA p34 subunit, **(b)** XPG protein and TFIIH subunits XPB and p62, **(c)** XPC protein, **(d)** XPA protein, or **(e)** XPF and ERCC1 proteins.

Figure 3



(a) Addition of purified XPA protein to dual incision reactions containing 50 ng DNA with the Pt-GTG adduct and 40 μg cell extract protein. Purified XPA (10–160 ng) was added where indicated to protein extracts from MGH-U1, 833K, GCT27 and control XPA-defective GM2345 cells. Extracts were incubated with Pt-GTG (50 ng) in the absence or presence of XPA protein as indicated. The region of the gel with the dual incision products is shown. **(b)** Addition of purified ERCC1–XPF complex to dual incision reactions containing 50 ng DNA with Pt-GTG adduct and 40 μg protein extract. Purified ERCC1–XPF complex (5–20 ng) was added to MGH-U1 and 833K extracts, or to control extracts from ERCC1-defective 43-3B cells. **(c)** NER dual incision by testis tumour cell extracts after addition of combinations of XPA, ERCC1–XPF and XPG proteins. Reaction mixtures included 40 μg HeLa (H), MGH-U1 (M), GCT27 or 833K cell extract protein and 50 ng DNA containing the Pt-GTG adduct, supplemented with XPA protein (40 ng), ERCC1–XPF complex (8 ng) or XPG protein (42 ng) as indicated (+). See Supplementary material published with this paper on the internet for quantification of these data.

core of the NER machinery and interacts with damaged DNA as well as with the other NER factors RPA, TFIIH and ERCC1 [4,5]. Human cells with inactivating mutations in the *XPA* gene [10] or knockout mice with disruptions in *XPA* [11,12] have absolutely no capacity for NER and are defective in removing cisplatin lesions from their genomes [9,13]. Coupled with other data indicating that the amount of XPA protein expressed in fibroblasts is a limiting factor for NER capacity [14–16], it is reasonable to expect that the amount of XPA in testis tumour cells significantly influences cisplatin sensitivity.

Cell lines derived from human testicular germ cell tumours retain their sensitivity to many different chemotherapeutic drugs and to radiation, reflecting the clinical response [17]. Many potential sensitising mechanisms have been investigated. For cisplatin these include not only DNA repair but intrinsic permeability to the drug, levels of intracellular scavengers, and pathways which promote apoptosis. For example, testis tumour cells (including those used in this study) contain high levels of non-mutated p53 and it has been suggested that the sensitivity to a broad range of drugs and radiation is largely due to a propensity to undergo apoptosis after DNA damage [18,19]. Other studies have concluded that there is no correlation between cisplatin sensitivity and p53 status of testis tumour cells [20,21].

Although the XPA protein is present in reduced amounts in the testis tumour cell lines, the level at which this is controlled is not known. Tissue-specific variations in XPA mRNA levels may exist and it has been reported that expression of XPA mRNA in normal mammalian testis tissue is relatively low [22], raising the possibility that even normal testis tissue has a low level of XPA protein and hypersensitivity to cisplatin. This requires further investigation with additional testis tumour cell lines and normal tissues. Overexpression of the relevant genes in testis tumour cell lines will give a fuller understanding of the involvement of NER factors in cellular hypersensitivity.

These observations further suggest that specific inhibition of XPA could sensitise other types of tumours to cisplatin and thereby broaden the usefulness of its class of chemotherapeutic agents. XPA is a unique potential target for chemotherapy amongst the core NER factors because it has no known involvement in other cellular pathways. XPA knockout mice, for example, are only distinguishable from normal mice by their sensitivity to UV light and chemical carcinogens [11,12]. This apparently unique role of XPA is in sharp contrast to the components RPA, TFIIH, XPG and ERCC1–XPF, each of which has some other function in a separate process of DNA replication, transcription, recombination, or another repair pathway [5,23]. Cellular reduction of XPA protein levels should therefore specifically reduce NER without affecting other aspects of DNA metabolism. Targeted inhibition of the activity of XPA in repair might be achieved, for example, by using a short peptide or small molecule inhibitor to disrupt either the critical XPA–RPA interaction or the binding of XPA to damaged DNA.

Supplementary material

Quantification of the data from Figure 3 and additional methodological details are published with this paper on the internet.

Acknowledgements

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Supplementary material

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Table S1

Quantification of data.*

Quantification of data from Figure 3a

XPA protein (ng)	MGH-U1	833K	GCT27
–	1.0	1.0	1.0
10	1.0	4.1	3.3
20	1.1	3.5	2.8
40	1.4	4.8	3.8
80	1.5	4.5	2.8
160	1.5	4.8	2.8

Quantification of data from Figure 3b

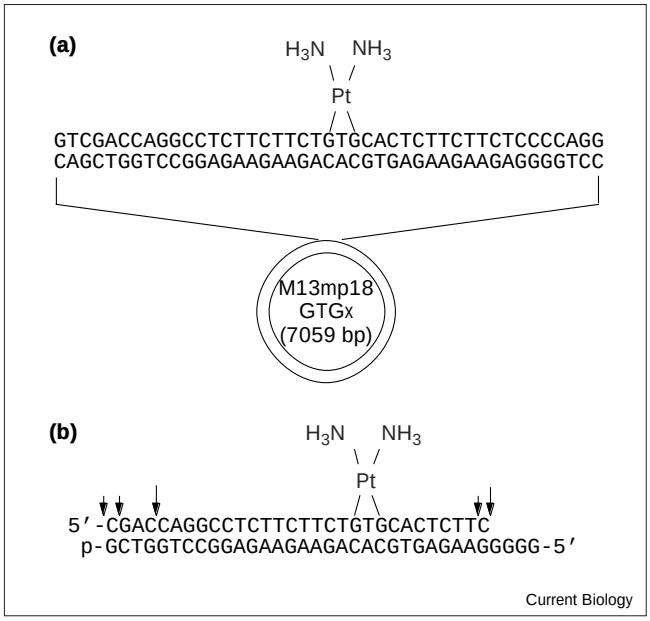
ERCC1–XPF complex (ng)	MGH-U1	833K	43-3B†
–	1.0	1.0	(1.0)
5	1.5	1.8	4.6
10	1.7	2.5	6.4
20	1.7	2.5	12.1

Quantification of data from Figure 3c

Purified protein	GCT27	833K‡
–	1.0	1.0
XPA	6.0	2.3
ERCC1–XPF	1.8	1.0
XPA, ERCC1–XPF	8.8	3.7
XPG	1.4	1.0
XPA, ERCC1–XPF, XPG	11.1	4.3

*Intensities of dual incision products were measured from autoradiographs using ImageQuant software. The values are the fold-stimulation of repair by addition of protein, after correction for local background. †Relative to background level. ‡Values in this column are underestimates of the stimulation because of the high background in lane 9.

Figure S1



(a) Closed circular M13mp18GTGx DNA duplex containing a single 1,3-intrastrand d(GpTpG) cisplatin cross-link within the sequence context shown. (b) To measure the dual incision reaction of NER, the DNA (or control duplex without an adduct) is incubated with cell extract for 30 min. As indicated by the arrows, the major 3′ incision sites are 8 and 9 phosphodiester bonds from the 3′ side of this adduct and 16, 19 and 20 phosphodiester bonds on the 5′ side. The excision products are annealed to a complementary oligonucleotide with a 3′ phosphate and additional G residues on the 5′ end, and directly labeled with [α-^{32}P]dCTP by DNA polymerase. The excision products are measured after separation on a DNA sequencing gel. Arrows in Figure 1a point to the position of the bands arising from cleavage at the three major 5′ incision sites, which are the major products in HeLa cells. There are several reasons for a more complex pattern of bands spanning the region from 24–32 nt, as found for other adducts. There is a limited shortening of primary incision products in the extracts, the detection method involves end-filling by dCTP which may not always give full length products and could in theory give rise to three shorter bands for each primary product, and there are probably some minor 5′ incision sites. The results are quantified as the sum of the entire band pattern, which takes into account all of these factors.

Supplementary materials and methods

Cells and cell extracts

The human testis germ cell tumour lines GCT27 (testis primary, embryonal carcinoma) and 833K (testis abdominal metastasis, embryonal carcinoma, teratoma) and the bladder cancer cell line MGH-U1 (transitional-cell carcinoma) were described previously [S1]. Both testis tumour cell lines have high levels of functional p53 [S2] as well as levels of the mismatch repair proteins MSH2, MLH1 and PMS2 that are in the normal range (P. Maisuria and B.K., unpublished data). These cell lines and the ERCC1-defective cell line 43-3B [S3] were grown as monolayers in 175 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The lymphoblastoid cell line 705ori was from a repair-proficient donor [S4]. Repair properties of the lymphoblastoid GM2345 cell line from the XPA-defective patient XP2OS, and the lymphoblastoid AG08802 cell line from the XPG-defective patient XP20BE have been described [S5,S6]. These cell lines were grown in suspension in RPMI 1640 medium with 10% fetal calf serum. HeLa cells were grown in suspension in RPMI 1640 medium with 5% fetal calf serum. Whole cell extracts were prepared as described [S7]. Care was taken to insure that cells were in the logarithmic growth phase at the time of harvesting.

Nucleotide excision repair in vitro

Covalently closed circular DNA (Figure S1a) containing a single 1,3-intrastrand d(GpTpG) cisplatin crosslink (Pt-GTG) or lacking damage (Con-GTG) was produced as described, using M13mp18GTGx bacteriophage

DNA [S8]. Repair was carried out in 10 μl reaction mixtures with whole cell extract protein as indicated in a buffer containing 45 mM Hepes-KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine (di-Tris salt), 0.5 μg creatine kinase, 3.4% glycerol and 3.3 μg BSA. Following preincubation for 5 min at 30°C, 50 ng of Pt-GTG DNA or Con-GTG DNA was added and incubation continued for 30 min at 30°C. The reaction was stopped by rapid freezing, and 6 ng of a 34-mer oligonucleotide complementary to the excised DNA with extra G residues at the 5′ end was added (Figure S1b). The excision products were radiolabelled with 0.13 units Sequenase v2.0 DNA polymerase and 2 μCi [α-^{32}P]dCTP, separated on a denaturing 14% polyacrylamide gel and visualized by autoradiography as described [S9]. Results were quantified with a phosphorimager or by densitometry.

SDS–PAGE and immunoblots

Protein extracts were prepared by lysing 5×10^6 cells on ice in 40 μl of buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 (1% Triton X-100 for XPC), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 97 μg/ml PMSF. After 30 min incubation the cells were centrifuged at 14,000 × g for 20 min at 4°C, the supernatant was recovered and protein content determined by the Bradford method. For immunoblotting, protein extracts were separated on SDS 10% polyacrylamide gels and transferred to Immobilon P membranes. Primary rabbit polyclonal or mouse monoclonal antibodies were as follows: XPA, 1/10,000 dilution of polyclonal antibody CJ1, raised against recombinant human XPA protein (C.J. Jones, unpublished). XPC, 1/2,000 dilution of polyclonal antibody RW028 raised against residues 96–299 of human XPC protein (D. Batty, unpublished). XPG, 1/500 dilution of monoclonal antibody 8H7 (E. Evans and J. Steel, unpublished). XPB 1/1,000 dilution of monoclonal antibody 2G12 and p62, 1/10,000 of monoclonal antibody 3C9, both provided by J-M. Egly. RPA p34 subunit, 1/1,000 dilution of monoclonal antibody 34A [S10]. XPF, 1/5,000 dilution of polyclonal antibody RA1 raised against residues 571–905 of human XPF protein (L. Fullerton and R. Ariza, unpublished). ERCC1, 1/1,500 dilution of polyclonal antibody RW017 [S11]. The membranes were incubated with the primary antibody for 1 h, followed by incubation for 1 h with either 1/25,000 dilution of peroxidase-labelled anti-mouse IgG or 1/25,000 dilution of peroxidase-labelled anti-rabbit IgG (both from Sigma). For detection of XPC protein the secondary antibody was diluted to 1/75,000. Bands were revealed with an ECL chemiluminescence kit (Amersham).

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