A yeast DNA repair gene partially complements defective excision repair in mammalian cells

Clare Lambert, Linda B.Couto,
William A.Weiss, Roger A.Schultz,
Larry H.Thompson¹ and Errol C.Friedberg

Department of Pathology, Stanford University School of Medicine,
Stanford, CA 94305 and ¹Biomedical Sciences Division, Lawrence
Livermore National Laboratory, PO Box 5507, Livermore, CA 94550,
USA

Communicated by M.Radman

The *RAD10* gene of *Saccharomyces cerevisiae* is required for nucleotide excision repair of DNA. Expression of *RAD10* mRNA and Rad10 protein was demonstrated in Chinese hamster ovary (CHO) cells containing amplified copies of the gene, and *RAD10* mRNA was also detected in stable transfectants without gene amplification. Following transfection with the *RAD10* gene, three independently isolated excision repair-defective CHO cell lines from the same genetic complementation group (complementation group 2) showed partial complementation of sensitivity to killing by UV radiation and to the DNA cross-linking agent mitomycin C. These results were not observed when *RAD10* was introduced into excision repair-defective CHO cell lines from other genetic complementation groups, nor when the yeast *RAD3* gene was expressed in cells from genetic complementation group 2. Enhanced UV resistance in cells carrying the *RAD10* gene was accompanied by partial reactivation of the plasmid-borne chloramphenicol acetyltransferase (*cat*) gene following its inactivation by UV radiation. The phenotype of CHO cells from genetic complementation group 2 is also specifically complemented by the human *ERCC1* gene, and the *ERCC1* and *RAD10* genes have similar amino acid sequences. The present experiments therefore indicate that the structural homology between the yeast Rad10 and human Ercc1 polypeptides is reflected at a functional level, and suggest that nucleotide excision repair proteins are conserved in eukaryotes.

Key words: *Saccharomyces cerevisiae*/UV radiation/DNA repair/mammalian cells

Introduction

Recent studies with purified proteins overexpressed from cloned *uvr* genes have demonstrated an unexpected biochemical complexity for nucleotide excision repair of DNA in the prokaryote *Escherichia coli* (Seeberg, 1978; Sancar and Rupp, 1983; Yeung *et al.*, 1983, 1986; Caron *et al.*, 1985; Husain *et al.*, 1985; Van Houten *et al.*, 1986; Oh and Grossman, 1987). In contrast, there is essentially no information about the biochemistry of this process in higher organisms. Genetic studies in a number of eukaryotes suggest that nucleotide excision repair is considerably more complex than in *E.coli*. In the yeast *Saccharomyces*

cerevisiae at least 10 genetic loci are involved in this process (Friedberg, 1985, 1988). Additionally, six genetic complementation groups for nucleotide excision repair have been defined in Chinese hamster ovary (CHO) cells (Thompson *et al.*, 1987) and nine human genetic complementation groups have been determined from studies of the disease xeroderma pigmentosum (XP) (Cleaver, 1983).

It is not established to what extent the multiple genes identified in these eukaryotes represent overlapping functions for nucleotide excision repair. A number of the yeast genes have been isolated by molecular cloning and have been extensively characterized (see Friedberg, 1988). The demonstration of structural or functional conservation of excision repair genes in yeast and higher eukaryotes offers the potential for utilizing yeast genes (or antibodies to proteins expressed from them), as specific probes for homologous elements in higher cells. Additionally, *S. cerevisiae* is particularly amenable to genetic and molecular analysis. Hence, this organism may be an informative paradigm for understanding the biochemical complexity of nucleotide excision repair in mammalian cells.

Several examples of functional conservation between genes in yeast and higher eukaryotes have been reported. Lee and Nurse (1987) isolated a human homologue of the cell cycle control gene *CDC2* of *Schizosaccharomyces pombe* by screening a human cDNA library for phenotypic complementation of a *cdc2* mutant. The multistep pathway for sterol metabolism is highly conserved in yeast and human cells (Henry, 1982), and it has recently been demonstrated that mammalian cDNAs for HMG CoA reductase and for mevalonate kinase complement the phenotype of appropriate yeast mutants (J.Rine and G.W.Robinson, personal communication). Finally, it has been shown that subunits of the human protein CP1 that binds to CCAAT-containing transcription elements, are functionally interchangeable with subunits of the HAP2/HAP3 protein complex of *S.cerevisiae* (Chodosh *et al.*, 1988).

We are aware of a single example of functional complementation of mammalian cells by a cloned yeast gene. In that study DeFeo-Jones *et al.* (1985) transformed NIH/3T3 cells with a mutant form of the yeast *RAS^{sc}-1* gene. In the present report we demonstrate that a cloned nucleotide excision repair gene from *S.cerevisiae* previously shown to share structural similarity with a human repair gene (Van Duin *et al.*, 1986), partially complements defective nucleotide excision repair in CHO cells.

RAD10 is one of at least five genes required for damage-specific incision of DNA during nucleotide excision repair in *S.cerevisiae* (Reynolds and Friedberg, 1981; Wilcox and Prakash, 1981). The gene was isolated by phenotypic complementation of a *rad10* mutant (Prakash *et al.*, 1985; Weiss and Friedberg, 1985) and nucleotide sequencing revealed an open reading frame of 210 amino acids, expected to encode a polypeptide of 24 284 daltons (Reynolds *et al.*, 1985). The human DNA repair gene *ERCC1* was

isolated by a similar strategy, in this case by complementation of a CHO cell line defective in nucleotide excision repair (Westerveld *et al.*, 1984). Comparison of the amino acid sequences translated from the yeast *RAD10* gene and the human *ERCC1* cDNA revealed considerable similarity, suggesting homology between these two polypeptides (Van Duin *et al.*, 1986).

The *ERCC1* gene specifically complements the phenotype of CHO mutants from genetic complementation group 2 and has no effect on the phenotype of mutants from other groups (Van Duin *et al.*, 1988). We show here that introduction of the cloned *RAD10* gene into three independently derived CHO mutant cell lines from genetic complementation group 2 results in partial complementation of UV sensitivity and sensitivity to mitomycin C. In keeping with the phenotype of defective excision repair, CHO mutants from genetic complementation group 2 are defective in restoring expression of the plasmid-borne *E. coli cat* gene following inactivation of the gene by UV radiation. However, when these mutants carry the *RAD10* gene they are able to partially reactivate the *cat* gene. These results suggest that the *RAD10* and *ERCC1* genes encode proteins with related functions required for nucleotide excision repair of DNA.

Results

Stable integration and amplification of the *RAD10* gene

The UV sensitive, excision repair-defective CHO cell line UV20TOR was transfected with plasmid pRSVNEOIV-*RAD10* ligated to plasmid pSVR400 (containing the mouse *DHFR* gene) (Figure 1), or with plasmid pRSVNEOIV-*RAD10* alone. Control cells were transfected with the pRSVNEOIV vector, the vector ligated to pSVR400, or were untransfected. Stable transfectants were selected for resistance to the antibiotic geneticin (G418). Additionally, cells transfected with the *DHFR* gene were treated with amethopterin to promote amplification of the gene and flanking sequences (Alt *et al.*, 1976). This strategy was designed to promote co-amplification of the *RAD10* gene.

To verify the presence of the *RAD10* gene in stable transfectants with or without amplified DNA, total genomic DNA from selected cell lines was subjected to Southern analysis using a *RAD10*-specific probe (Figure 2, left). Quantitative dot-blot analysis showed the presence of ~6 copies of *RAD10* in the genome of a cell line (designated UV20NEORAD10) not exposed to the gene amplification protocol and ~140 copies of *RAD10* in a cell line (designated UV20LANEORAD10-RP5) treated with amethopterin (Figure 2, right). No *RAD10* signal was detected in DNA from control cell lines (Figure 2).

Expression of *RAD10* mRNA and Rad10 protein in CHO cells

Northern analysis using a *RAD10*-specific probe revealed the presence of a single ~1.3 kb transcript in the cell line UV20LANEORAD10-RP5 in which the *RAD10* gene was known to be amplified (Figure 3). *RAD10* transcripts were also detected in cell lines with non-amplified stably integrated copies of the gene (data not shown). No hybridization signal was detected in cells transfected with plasmids without the *RAD10* gene (Figure 3). Western analysis of nuclear (Figure 4) and cytoplasmic (data not shown) preparations of

Fig. 1. Plasmids used for transfection of CHO cells with the yeast *RAD10* gene. See text for experimental details.

Fig. 2. DNA hybridization analysis of transfected cells. **Left:** Southern analysis of total cellular DNA probed with ^{32}P -labelled *RAD10* DNA. Lanes 1–5, DNA digested with *Hind*III; lanes 6–9, DNA digested with *Eco*RI. Lanes 1, 2 and 6 contain DNA from three different cell lines in which the *RAD10* gene was amplified. Lanes 3 and 7 are from a stable transformant without amplified copies of *RAD10*. Lanes 4, 5, 8 and 9 are from control lines transfected with the vector pRSVNEOIV without *RAD10*. **Right:** Dot-blot analysis of total cellular DNA probed with ^{32}P -labelled *RAD10* DNA. 1, UV20TOR cells transfected with the vector pRSVNEOIV; 2, a cell line (UV20NEORAD10) stably transfected with the *RAD10*-containing plasmid; 3, a cell line transfected with the vector and exposed to the gene amplification protocol (see text for details); 4 and 5, a cell line (UV20LANEORAD10-RP5) transfected with the *RAD10*-containing plasmid and exposed to the gene amplification protocol.

UV20LANEORAD10-RP5 cells revealed a protein band of $M_r \sim 23.5$ kd — the expected size of Rad10 protein (Weiss and Friedberg, 1985; Reynolds *et al.*, 1985).

Phenotypic complementation of CHO cells

Functional activity of the yeast *RAD10* gene in mammalian cells was examined in several ways. In one set of experiments we quantitated the survival of cells exposed to various DNA-damaging agents. UV20LANEORAD10-RP5 cells showed enhanced resistance to killing following exposure to UV radiation (Figure 5). In contrast, no difference in UV

Fig. 3. Northern analysis of RNA from transfected cells. Total RNA was probed with ^{32}P -labelled *RAD10* DNA. Exposure was on Kodak XAR-5 film at -70°C for 2 h. Lanes 1, 3 and 4 contain RNA from three separate pools of cells transfected with the *RAD10*-containing plasmid and treated with amethopterin for varying periods. Lane 2 contains RNA from control cells transfected with the vector.

Fig. 4. Western blot of total nuclear proteins from UV20LANEORAD10-RP5 cells (lane 2) and from control cells transfected with the vector (lane 1). Nuclear fractions were probed with affinity-purified antiserum to Rad10 protein (see text for details). Rabbit antisera and rabbit preimmune sera reacted non-specifically with some proteins in both preparations. Additionally, some of the bands seen in both lanes resulted from non-specific reaction of CHO proteins with alkaline phosphatase-tagged biotin in the Vectastain immunodetection kit. The single band unique to lane 2 is identified by the arrow and has a mol. wt ($\sim 24\,000$) expected for Rad10 protein.

sensitivity was detected between untransfected UV20TOR cells and cells transfected with the plasmid vector alone (Figure 5). Enhanced resistance in these cells was strictly reproducible and was detected at all UV doses examined. However, complementation was clearly partial relative to the survival of wild-type CHO cells (Figure 5).

In order to quantitatively evaluate the extent of phenotypic complementation we compared the dose that reduces survival to 37% (D_{37}) in cells with and without *RAD10*.

Fig. 5. Complementation of the UV sensitivity of UV20TOR cells by the yeast *RAD10* gene. The cell line UV20LANEORAD10-RP5 contains amplified copies of the *RAD10* gene. Line UV20LANEOP5 is transfected with the vector pRSVNEOIV ligated to plasmid pSVR400 and was treated with amethopterin. UV20 is the untransfected UV20TOR lines. The error bars show the experimental variation between three replicate measurements. The survival of wild-type CHO AA8 cells determined in independent experiments is shown for comparison.

UV sensitivity was measured in an extensive series of experiments using 12 independently isolated cell lines transfected with the *RAD10* and *DHFR* genes and exposed to the gene amplification protocol. The difference in the D_{37} was essentially zero when comparisons were made between UV20TOR-derived cell lines with or without the plasmid vector (Table I). However, when comparing the parameter [D_{37} *RAD10* - D_{37} control] between control cells and those with amplified copies of the *RAD10* gene a difference was consistently noted, ranging between 0.25 and 0.60 J/m^2 (Table I). Thus, in every experiment the UV dose required to reduce the survival of *RAD10*-containing cells to 37% was greater than that required to reduce the survival of control cells to the same level. Table I also shows a comparison of the dose of UV radiation or mitomycin C required to yield identical 10% survival (D_0) values. The resulting dose modifying factors (DMF) range from 1.2 to 1.8 for cells transfected with the *RAD10* gene.

Complementation of the UV sensitivity of UV20TOR cells was not dependent on amplification of the *RAD10* gene. Comparable levels of enhanced UV resistance were observed in UV20 cell lines containing stably integrated, non-amplified copies of *RAD10* and following transfection of cells under conditions of transient expression of the gene (Table II). Complementation was also observed with two other independently isolated CHO mutants (UV4 and UV46) belonging to genetic complementation group 2 (Table II).

Partial phenotypic complementation of CHO cells by the

Table I. Phenotypic complementation of CHO group 2 cells by the amplified yeast *RAD10* gene

Cell lines compared	DNA-damaging agent	Complementation [D ₃₇ radio – D ₃₇ control] (J/m ² or ng/ml MMC)	DMF ^d
UV20NEOP5-UV20TOR	UV	–0.04	1
UV20NEO2II-UV20TOR	UV	0	1
RP5(<i>RAD10</i>)-UV20TOR	UV	0.60 ^a	1.5
RP5(<i>RAD10</i>)-UV20 NEOP5	UV	0.60	1.5
RP5-1(<i>RAD10</i>)-UV20 NEOP4	UV	0.50	1.3
RP5-4(<i>RAD10</i>)-UV20 NEOP4	UV	0.55 ^b	1.8
LA(<i>RAD10</i>)-UV20 NEO2II	UV	0.55 ^b	1.4
LA(<i>RAD10</i>)-UV20 NEO2III	UV	0.25	1.2
2II(<i>RAD10</i>)-UV20TOR	UV	0.40	1.4
2II(<i>RAD10</i>)-UV20 NEO2II	UV	0.45	1.3
2II(<i>RAD10</i>)-UV20 NEOLA	UV	0.40	1.5
2III(<i>RAD10</i>)-UV20 NEO2IV	UV	0.55 ^c	1.6
2III(<i>RAD10</i>)-UV20 NEO2II	UV	0.55 ^b	1.4
LA(<i>RAD10</i>)-UV20 NEOLA	MMC	6.3 ^b	1.5

^aFrom Figure 5.^bAverage of three experiments.^cAverage of seven experiments.^dDose modifying factor at 10% survival (D₀).

yeast *RAD10* gene is specific to mutants from genetic complementation group 2. No enhanced UV resistance was observed in excision-defective CHO cell lines from genetic complementation groups 1 (UV5), 3 (UV24), 4 (UV41) or 5 (UV135), examined under conditions of either amplification (Figure 6A and Table II), transient expression (Figure 6B,C and Table II), or stable transfection of the *RAD10* gene (Table II).

CHO mutants defective in nucleotide excision repair are also abnormally sensitive to chemicals that produce bulky base adducts in DNA, including DNA cross-linking agents such as mitomycin C (Hoy *et al.*, 1985; Thompson *et al.*, 1987). UV20TOR cells containing amplified copies of *RAD10* showed partial complementation of this phenotype as well, at levels comparable to that observed following exposure to UV radiation (Figure 7A and Table I). On the other hand, cells from genetic complementation group 2 are not abnormally sensitive to killing by ionizing radiation or chemicals that produce strand breaks in DNA (Thompson *et al.*, 1980). Consistent with this observation, lethality from gamma radiation was unaffected in cells containing the *RAD10* gene (Figure 7B).

To confirm further the specificity of phenotypic complementation we transfected CHO mutants with a yeast excision repair gene other than *RAD10*. The *RAD3* gene [another of the five *RAD* genes required for specific incision of DNA during nucleotide excision repair (Friedberg, 1988)] had no effect on the UV resistance of UV20TOR cells (Figure 6D). In independent experiments we established that *RAD3* mRNA was expressed in these cells (data not shown). The yeast *RAD3* gene also had no effect on the UV sensitivity of excision-defective CHO mutants from genetic complementation groups 3, 4 and 5 (data not shown). Experiments with cells from complementation group 1 are still in progress.

The specificity of phenotypic complementation in terms of a particular CHO genetic complementation group, in terms of particular forms of DNA damage and in terms of

Table II. Phenotypic complementation of CHO cells from different genetic complementation groups by the yeast *RAD10* gene

CHO complementation group (CG)	Expression strategy	Complementation [D ₃₇ radio – D ₃₇ control] (J/m ²)
CG2	Amplification	0.52 ^a
CG2	Stable integration	0.34
CG2 ^b	Stable integration	0.45
CG2 ^c	Transient expression	0.40
CG1	Transient expression Stable integration	–0.1
CG3	Stable integration	0.04
CG4	Stable integration	0
CG5	Amplification Stable integration	0.06

^aData are the average of the experiments shown in Table I.^bThese experiments involved the excision defective CHO mutant UV4 from genetic complementation group 2.^cThese experiments involved the excision defective CHO mutants UV46 and UV4 from genetic complementation group 2.

particular yeast *RAD* genes, argues against a trivial explanation for this result. Consistent with this interpretation, we noted no significant differences in the kinetics of growth of complemented and control cell lines, and phenotypic complementation was observed under conditions of comparable chromosomal ploidy and cell size. We therefore conclude that partial complementation of CHO cells of genetic complementation group 2 is specifically associated with expression of the yeast *RAD10* gene.

DNA repair in complemented cell lines

Reactivation of the *E.coli* chloramphenicol acetyltransferase

Fig. 6. Effect of the *RAD10* or *Rad3* genes on the UV sensitivity of various CHO cell lines. (A) UV135RAD10MTX is a cell line isolated following transfection of UV135 cells (genetic complementation group 5) and contains amplified copies of the *RAD10* gene. Line UV135NEOMTX is a control line containing amplified vector sequences without *RAD10*. (B) UV135+RAD10 and UV135+NEO are stable transfectants of UV135. The former contains non-amplified copies of *RAD10*. (C) UV5+RAD10 is a cell line from genetic complementation group 1 which was stably transfected with the *RAD10* gene. UV5+NEO is a control line. (D) UV20+RAD3 is UV20TOR transiently transfected with a plasmid containing the *RAD3* gene. UV20+NEO is a control cell line.

(*cat*) gene carried on a non-replicating plasmid vector has been shown to be a sensitive and specific end-point for measuring nucleotide excision repair in human cells (Kraemer *et al.*, 1987). To determine whether enhanced resistance to UV radiation associated with transfection of UV20TOR cells with the *RAD10* gene was accompanied by increased excision repair of DNA, we measured reactivation of *cat* gene function following its inactivation by UV radiation. In initial experiments we transfected the wild-type CHO line AA8 with unirradiated or UV irradiated plasmid DNA. Post-irradiation incubation of the transfected cells resulted in complete restoration of *cat* gene activity relative to unirradiated plasmids (Figure 8 and Table III). In contrast, excision-defective UV20TOR cells demonstrated very low levels of *cat* gene activity following transfection with plasmids irradiated at the same dose (Figure 8 and Table III).

Having established the utility of the *cat* gene assay in CHO cells we measured chloramphenicol acetyltransferase activity in various UV20TOR isolates containing either amplified or

Fig. 7. Top panel: complementation of sensitivity to mitomycin C by the *RAD10* gene. UV20MTXRAD10LA contains amplified copies of *RAD10*. UV20MTXNEOLA contains amplified vector sequences without *RAD10*. The error bars show the experimental variation between three replicate measurements. Bottom panel: the *RAD10* gene does not affect the sensitivity of UV20TOR cells to gamma radiation. NEORAD10LA and NEORAD10211 are independently isolated UV20TOR cell lines containing amplified copies of *RAD10*. NEOLA and NEO211 are the respective controls without *RAD10*.

stably integrated non-amplified copies of the *RAD10* gene. Consistent with the earlier demonstration of increased resistance to killing by UV radiation, transfected cells showed enhanced *cat* gene activity relative to cells without

Fig. 8. Reactivation of the plasmid-borne *E.coli cat* gene in CHO cells. AA8 (repair-proficient) and UV20TOR (repair-defective) cells were transfected with identical aliquots of unirradiated or UV irradiated (800 J/m²) pRSVcat plasmid DNA. Following incubation, extracts were prepared and assayed for Cat activity as described in the text. The autoradiogram shows the conversion of [¹⁴C]chloramphenicol (bottom row of spots) to mono- and di-acetylated forms (middle and upper row of spots respectively) in AA8 cells transfected with either unirradiated or UV irradiated plasmids. UV20TOR cells failed to reactivate the gene in the UV irradiated plasmids.

the *RAD10* gene (Figure 9 and Table III). In each experiment Cat activity in cell lines transfected with UV irradiated plasmids was normalized against the level of enzyme activity in cells transfected with unirradiated plasmids, to account for possible differences in transfection efficiency (Table III). A comparison of the normalized levels of Cat activity between cells with and without *RAD10* showed a 2- to 10-fold enhancement following irradiation at a UV dose of 800 J/m² (Table III). *RAD10*-dependent reactivation of the *cat* gene was also observed following transfection with plasmids irradiated at 600 J/m² (data not shown).

The presence of amplified copies of the *RAD10* gene in UV135 cells (genetic complementation group 5) had no effect on the level of Cat expression. Hence, reactivation of the *cat* gene in CHO cells from genetic complementation group 2 is not the result of a catalytic function of Rad10 protein that bypasses the endogenous CHO nucleotide excision repair system during repair of plasmid DNA.

Discussion

The *RAD10* gene of *S.cerevisiae* is absolutely required for

Table III. Reactivation of UV irradiated plasmid DNA measured by the Cat assay

Cell line	Cat activity/10 ⁶ cells (% of [¹⁴ C]chloramphenicol acetylated)		Normalized Cat activity $\frac{\text{UV}}{\text{No UV}} \times 100$	<i>cat</i> gene reactivation factor
	No UV	UV		
AA8	92	94	102.1	
	90	95	105.5	
UV20TOR	70	5.2	7.4	1.0
	68	4.7	6.9	
UV20RAD10	80	10.6	13.3	1.8
	93	11.6	12.5	
UV20NEO	65	2.4	3.7	1.0
UV20NEORAD10	46	4.0	9.0	2.4
MTXNEO	30	0.5	1.7	1.0
		0.6	1.9	
MTXNEORAD10	94	7.0	7.3	3.6
		5.0	5.5	
UV20NEO	66	0.7	1.1	1.0
RP5-1	93	12.0	13.0	9.5
		8.0	8.0	
LAMTXNEO	45	3.8	8.4	1.0
LAMTXNEORAD10	44	22.5	51.1	6.0
		20.0	45.4	
UV135MTXNEO (CG5)	58	9	1.6	1.0
		10	1.7	
UV135MTXNEORAD10 (CG5)	70	5	0.7	0.73
		12	1.7	

Chloramphenicol acetyltransferase (Cat) activity was measured as described in Materials and methods. The cell line AA8 is repair-proficient, while the cell lines UV20TOR, UV20NEO, MTXNEO and LAMTXNEO are defective in nucleotide excision repair (genetic complementation group 2) and were either untransfected (UV20TOR) or transfected with control plasmids that do not contain *RAD10*. The cell lines UV20RAD10, MTXNEORAD10, RP5-1 and LAMTXNEORAD10 (genetic complementation group 2) contained amplified copies of the *RAD10* gene. The cell lines MTXNEO and LAMTXNEO were amplified concurrently with MTXNEORAD10, and LAMTXNEORAD10, but contain just vector sequences. UV20NEORAD10 (genetic complementation group 2) is a stable transfectant containing non-amplified copies of *RAD10*. UV135MTXNEO is an excision defective line (genetic complementation group 5), which contains amplified copies of plasmid pRSVNEOIV. UV135MTXNEORAD10 additionally contains amplified copies of the *RAD10* gene. In all experiments with cells not containing *RAD10* the *cat* gene reactivation factor is normalized to 1.0.

the excision of bulky base adducts from DNA. Deletion mutants are highly sensitive to UV radiation (Weiss and Friedberg, 1985) and are defective in the incision of UV irradiated DNA *in vivo* (Reynolds and Friedberg, 1981; Wilcox and Prakash, 1981). A similar phenotype is displayed by mutants defective in other yeast *RAD* genes, including the *RAD3* gene used in the present study (Reynolds and Friedberg, 1981; Wilcox and Prakash, 1981). These observations suggest that the products of at least five *RAD* loci function as a multiprotein complex for incision of damaged DNA, and possibly for coupled excision of damaged nucleotides (Friedberg, 1988).

A requirement for a multiprotein complex for nucleotide excision repair has been demonstrated in *E. coli* (Seeberg, 1978; Sancar and Rupp, 1983; Yeung *et al.*, 1983). The genetic complexity of nucleotide excision repair in human cell lines derived from the disease XP (Cleaver, 1983) and in laboratory-derived mutant strains of CHO cells (Thompson *et al.*, 1981, 1987; Thompson and Carrano, 1983) suggests that multiprotein DNA repair complexes are also utilized in higher eukaryotes. In addition to the possible requirement for multiple proteins to constitute a catalytically active repair endonuclease, components of such complexes may have important roles in processes that alter chromatin conformation, thereby facilitating access of excision repair enzymes to sites of base damage in the genome. The recent demonstration that the protein encoded by the *RAD6* gene of *S. cerevisiae* is a ubiquitin-conjugating enzyme with specificity for particular histones, hints at a possible role of DNA repair proteins in chromatin remodelling (Jentsch *et al.*, 1987).

Examination of the amino acid sequences of the cloned *RAD1*, *RAD2*, *RAD3*, *RAD4* and *RAD10* genes of *S. cerevisiae* did not reveal extended regions of similarity with the *E. coli* *uvrA*, *uvrB* or *uvrC* genes (Friedberg, 1988). On the other hand, the human DNA repair gene *ERCC1* shares considerable amino acid sequence similarity with *RAD10*. In some domains 50% of the amino acids are identical or closely related, and overall the similarity is ~30% (Van Duin *et al.*, 1986).

The *ERCC1* gene was isolated by phenotypic complementation of CHO excision repair mutants from genetic

complementation group 2 (Westerveld *et al.*, 1984; Rubin *et al.*, 1985). In the present studies we have demonstrated that the yeast *RAD10* gene is expressed in CHO mutants from this genetic complementation group following transfection of cells with plasmid DNA. Transfectants of three independently isolated mutant cell lines from genetic complementation group 2 showed partial complementation of sensitivity to UV radiation and to mitomycin C. This was observed in transfectants containing amplified copies of the *RAD10* gene as well as in stable transfectants containing non-amplified copies of the gene. The phenomenon was also observed in cells under conditions in which plasmid-borne genes are known to be transiently expressed.

In addition to complementation of lethality, cells containing the yeast *RAD10* gene showed partial reactivation of the *E. coli* *cat* gene following its inactivation by UV radiation. Reactivation of this gene correlated with the previously established ability of CHO cells to carry out nucleotide excision repair of DNA. Hence, we conclude that the phenomenon reflects repair of the *cat* gene in a fraction of the irradiated plasmids, and that enhanced UV (and mitomycin C) resistance observed in mutant CHO cells harboring the yeast *RAD10* gene reflects functional complementation of nucleotide excision repair.

Our results indicate that reactivation of UV inactivated plasmid-borne *E. coli* *cat* gene is an extremely sensitive assay for monitoring excision repair of DNA in mammalian cells, since we were not able to observe increased DNA repair by measuring unscheduled DNA synthesis or damage-specific incision of DNA (by alkaline elution analysis) (data not shown). These results are entirely consistent with the limited enhancement of survival of cells treated with UV radiation or mitomycin C, and suggest that one or more parameters of nucleotide excision repair are limiting in the transfected cells. Rad10 protein was detected in nuclear extracts of transfected CHO cells. However, at present no assay is available to evaluate the functional activity of the protein. Additionally, we cannot exclude the possibility that Rad10 protein is degraded in mammalian cells. Indeed, such a possibility is consistent with the observation that *RAD10* gene copy number did not influence the extent of phenotypic complementation. Even if the amount or specific activity of Rad10 protein was not limiting, the predicted amino acid sequence of this protein and of the Ercc1 protein are not identical, and hence one would not expect the yeast protein to be perfectly accommodated in a putative mammalian excision repair complex.

The results obtained in this study must be distinguished from those reported by Valerie *et al.* (1985, 1987) in which expression of the cloned *denV* gene of bacteriophage T4 was shown to complement defective excision repair in CHO and in human XP cells. The *denV* gene encodes a pyrimidine dimer-specific DNA glycosylase (Radany and Friedberg, 1980) that initiates excision repair of pyrimidine dimers by a mechanism that is biochemically distinct from nucleotide excision repair. Thus, phenotypic complementation by expression of the *denV* gene is not specific to particular mutant cell lines (Valerie *et al.*, 1985, 1987) as in the case of the yeast *RAD10* gene, and does not represent an example of functional conservation between bacteriophage and mammalian repair genes.

The *RAD6* gene is also involved in cellular responses to DNA damage in yeast (Haynes and Kunz, 1981; Lawrence,

Fig. 9. Reactivation of the plasmid-borne *E. coli* *cat* gene in UV20LANEORAD10-RP5 cells (designated UV20RAD10) containing amplified copies of the *RAD10* gene. The control line is UV20TOR. Experiments were carried out as described in the legend to Figure 8. The figure shows the results obtained in duplicate experiments with UV irradiated plasmids.

1982). As indicated above, it is now known that *RAD6* encodes a ubiquitin conjugating enzyme, and this gene is highly conserved in mammals (Jentsch *et al.*, 1987). The demonstration of both structural and functional relationships between two yeast and mammalian proteins involved in DNA repair indicates that some of these proteins have been conserved during eukaryote evolution and suggests that such conservation may be general. Given the problems associated with cloning human DNA repair genes by currently available strategies (Thacker, 1986), studies in yeast may facilitate the isolation of specific DNA or antibody probes for homologous genes and proteins in higher cells.

Materials and methods

Cell lines and culture

The UV resistant CHO cell line AA8 (Thompson *et al.*, 1987) and the UV sensitive, excision repair-defective lines UV20TOR, UV4 and UV46 [complementation group 2 (Thompson *et al.*, 1981; Hoy *et al.*, 1985)], UV5 [complementation group 1 (Thompson *et al.*, 1981)], UV24 [complementation group 3 (Busch *et al.*, 1980)], UV41 [complementation group 4 (Busch *et al.*, 1980)] and UV135 [complementation group 5 (Thompson and Carrano, 1983)], were maintained in minimal Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were grown in Corning plastic flasks and incubated at high humidity in incubators containing ~5% CO₂ in air. Stable transfectants were maintained in 10% MEM containing G418 (Geneticin) at 400 µg/ml. Cells with amplified *DHFR* sequences were maintained in methotrexate (2–10 mM) in MEM supplemented with 10% dialysed FCS.

Plasmids and DNA preparations

Plasmid pRSVNEOIV was constructed by cloning the *neo* gene as well as the SV40 splice site and polyadenylation signal from plasmid pSV2_{neo} into plasmid pRSV4 (generously provided by Dr Gilbert Chu, Stanford University). The yeast *RAD10* gene was inserted into this plasmid at a *Sal*I site in the polylinker region of pRSVNEOIV. Plasmid pSVR400 containing a cDNA copy of the mouse *DHFR* gene was a gift from Dr Christian Simonsen, Invitron Corp., Redwood City, CA. Plasmid pRSVcat was originally constructed by Gorman *et al.* (1982) and was obtained from Dr G. Crabtree, Stanford University. Plasmids were propagated in *E. coli* HB101. Plasmid DNA was isolated and purified as previously described (Clewell and Helinski, 1969).

Genomic DNA was isolated from ~10⁸ cells suspended in STE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl), plus 1 ml 10% SDS. The mixture was successively incubated with 100 µg/ml proteinase K, 100 mg RNase A and proteinase K (100 µg/ml). DNA was purified with phenol/chloroform, spooled into TE buffer and stored in buffer plus 1% sodium azide at a concentration of 250 µg/ml.

DNA transfection

For stable transfection recipient cells (5 × 10⁵) were plated in 100-mm dishes for at least 2 h before transfection with plasmid DNA (10 µg/dish), using the polybrene procedure described by Kawai and Nishizawa (1984). For transient gene expression cells were transfected by the calcium phosphate co-precipitation procedure of Wigler *et al.* (1979) and examined for gene expression 48 h later.

Gene amplification

Stable G418^R cells were initially selected for resistance to varying levels of amethopterin (methotrexate; Sigma) (0.2, 0.3 or 0.4 mM) as previously described (Alt *et al.*, 1976).

DNA–DNA hybridization analysis

Cellular DNA from stably transfected cells and from cells subjected to gene amplification by treatment with methotrexate was probed for *RAD10* sequences by Southern hybridization as described (Southern, 1975). To quantitate amplification of *RAD10* sequences 10 µg of denatured DNA from different cell lines was spotted onto a filter and hybridized against an excess of ³²P-labelled *RAD10* probe. Amplification was determined by measuring the radioactivity bound in each spot relative to that which hybridized to known amounts of purified *RAD10* DNA.

Northern analysis of *RAD10* mRNA

Transfected cells were lysed in 24% (w/v) sucrose with 1% NP-40 in the presence of 10 mM vanadyl ribonucleosides. Total RNA was isolated and electrophoresed in 1.2% agarose–formaldehyde gels. Northern analysis was carried out according to the instructions of the manufacturer of GeneScreen (New England Nuclear), using ³²P-labelled *RAD10* DNA as a probe.

Detection of *Rad10* protein by Western analysis

Nuclear and cytoplasmic protein fractions were isolated from CHO cells transformed with plasmids containing the *RAD10* gene, and from control transformants without *RAD10*. Cells were lysed and nuclear and cytoplasmic fractions were isolated. These fractions were precipitated with 5% TCA, resuspended and boiled in cracking buffer (Sancar *et al.*, 1981) and electrophoresed in 12% SDS–polyacrylamide gels. The gels were electroblotted to nitrocellulose (Schleicher and Schuell, Keene, NH). Transfers were blocked in a solution containing 1% dried non-fat milk, and were incubated with affinity purified antiserum raised against *Rad10* protein overexpressed in *E. coli* (W.A. Weiss and E.C. Friedberg, unpublished data). The antiserum was purified by precipitation in 40% ammonium sulfate, followed by preadsorption against an extract of total CHO cellular protein coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Sweden). Bands were visualized using the Vectastain ABC alkaline phosphatase kit (Vector Systems, Burlingame, CA).

Measurement of sensitivity to DNA damaging agents

All survival curves were determined with cells in exponential growth phase (1–5 × 10⁵ cells/ml). Treatment with mitomycin C was for 90 min at 37°C using a buffered stock solution at 10^{−5} M. Cells were exposed to UV light at 254 nm at a dose rate of 0.5 J/m²/s or 0.12 J/m². Shielding was minimized by removing cells from the sides of the dishes with a sterile swab. Following irradiation cells were plated in 100 mm dishes at various dilutions for quantitation of survival. For gamma irradiation cells were exposed at a dose rate of 1–3 krad/min. After treatment plates were incubated for 8–12 days at 37°C in MEM containing 10% FCS. Plates were left undisturbed to prevent cells from generating satellite colonies. To quantitate survival colonies were fixed with 100% ethanol for 30 min and stained with 2.5% Giemsa stain in 2.5% methanol.

Reactivation of the UV irradiated *cat* gene

Cells (10⁶/plate) were transfected with identical aliquots of UV irradiated or unirradiated pRSVcat plasmid DNA and after 40 h of incubation crude extracts were prepared by sonication. Extracts containing the same amount of protein were assayed for Cat activity as described by Gorman *et al.* (1982) using 3 mM acetyl CoA and 5 µCi of [¹⁴C]chloramphenicol (~200 000 c.p.m.), except that incubations were at 56°C instead of 37°C, as suggested by Klocker *et al.* (1985). Substrate and reaction products were visualized by autoradiography and quantitated by liquid scintillation counting. Reactivation of the *cat* gene was calculated as the percentage of radiolabelled chloramphenicol converted to acetylated products in extracts of cells transfected with UV irradiated plasmid, relative to that in extracts of cells transfected with unirradiated plasmid.

Acknowledgements

We thank Drs Robert Schiurba and David Goldfarb for valuable experimental advice, Drs Paul Berg and Gilbert Chu for plasmid pRSV4, Dr Christian Simonsen for plasmid pSVR400, and colleagues in our laboratory for stimulating discussions and critical review of the manuscript. These studies were supported by research grant CA-12428 from the USPHS (E.C.F.), and by DOE contract W-7045-ENG-48 to the Lawrence Livermore National Laboratory (L.H.T.). C.L. is supported by postdoctoral fellowship B/RF/7943 from SERC, UK, L.B.C. was supported by NRSA grant CA-07705 from the USPHS, and W.A.W. was supported by training grant CA-09302 from the USPHS.

References

- Alt, F., Kellerns, R.E., Berhiro, J.R. and Schimke, R.T. (1976) *J. Biol. Chem.*, **253**, 1357–1370.
- Busch, D.B., Cleaver, J.E. and Glaser, D.A. (1980) *Somatic Cell Genet.*, **6**, 407–418.
- Caron, P.R., Kushner, S.R. and Grossman, L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4925–4929.

- Chodosh, L.A., Olesen, J., Hahn, S., Baldwin, A.S., Guarente, L. and Sharp, P.A. (1988) *Cell*, **53**, 25–35.
- Cleaver, J.E. (1983) In Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L. and Brown, M.S. (eds), *The Metabolic Basis of Inherited Disease*. McGraw-Hill, New York, pp. 1227–1248.
- Clewell, D.B. and Helinski, D.R. (1969) *Proc. Natl. Acad. Sci. USA*, **62**, 1159–1166.
- DeFeo-Jones, D., Tatchell, K., Robinson, L.C., Sigal, I.S., Vass, W.C. and Scolnick, E.M. (1985) *Science*, **228**, 179–184.
- Friedberg, E.C. (1985) *Cancer Surveys*, **4**, 529–536.
- Friedberg, E.C. (1988) *Microbiol. Rev.*, **52**, 70–102.
- Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
- Haynes, R.H. and Kunz, B.A. (1981) In Strathern, J.N., Jones, E.W. and Broach, J.R. (eds), *The Molecular Biology of the Yeast *Saccharomyces*. Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 361–414.
- Henry, S.A. (1982) In Strathern, J.N., Jones, E.W. and Broach, J.R. (eds), *The Molecular Biology of the Yeast *Saccharomyces*. Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 101–158.
- Hoy, C.A., Thompson, L.H., Mooney, C.L. and Salazar, E.P. (1985) *Cancer Res.*, **45**, 1737–1743.
- Husain, I., Van Houten, B., Thomas, D., Abdel-Monem, M. and Sancar, A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6774–6776.
- Jentsch, S., McGrath, J.P. and Varshavsky, A. (1987) *Nature*, **329**, 131–134.
- Kawai, S. and Nisgizawa, M. (1984) *Mol. Cell. Biol.*, **4**, 1172–1174.
- Klocker, H., Schneider, R., Burstcher, J.H., Auer, B., Hirsch-Kaufmann, M. and Schweiger, M. (1985) *Eur. J. Cell Biol.*, **39**, 346–351.
- Kraemer, K.H., Protic-Sabljic, M., Bredberg, A. and Seidman, M.M. (1987) *Curr. Probl. Dermatol.*, **17**, 166–181.
- Lawrence, C.W. (1982) *Adv. Genet.*, **21**, 173–254.
- Lee, M.G. and Nurse, P. (1987) *Nature*, **327**, 31–35.
- Oh, E. and Grossman, L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3638–3642.
- Prakash, L., Dumais, D., Polakowska, R., Perozzi, G. and Prakash, S. (1985) *Gene*, **34**, 55–61.
- Radany, E.H. and Friedberg, E.C. (1980) *Nature*, **286**, 182–185.
- Reynolds, R.J. and Friedberg, E.C. (1981) *J. Bacteriol.*, **146**, 692–704.
- Reynolds, P., Prakash, L., Dumais, D., Perozzi, G. and Prakash, S. (1985) *EMBO J.*, **4**, 3549–3552.
- Rubin, J.S., Prideaux, V.R., Willard, H.F., Dulharty, A.H., Whitmore, G.F. and Bernstein, A. (1985) *Mol. Cell. Biol.*, **5**, 389–405.
- Sancar, A. and Rupp, W.D. (1983) *Cell*, **33**, 249–260.
- Sancar, A., Clarke, N.D., Griswold, J., Kennedy, W.J. and Rupp, W.D. (1981) *J. Mol. Biol.*, **148**, 63–76.
- Seeberg, E. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2569–2573.
- Southern, E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
- Thacker, J. (1986) *Int. J. Radiat. Biol.*, **50**, 1–30.
- Thompson, L.H. and Carrano, A.V. (1983) In Friedberg, E.C. and Bridges, B.A. (eds), *Cellular Responses to DNA Damage*. Alan R. Liss, New York, pp. 125–143.
- Thompson, L.H., Rubin, J.S., Cleaver, J.E., Whitmore, G.F. and Brookman, K. (1980) *Somat. Cell Genet.*, **6**, 391–405.
- Thompson, L.H., Busch, D.B., Brookman, K., Mooney, C.L. and Glaser, D.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3734–3737.
- Thompson, L.H., Salazar, E.P., Brookman, K.W., Collins, C.C., Stewart, S.A., Busch, D.B. and Weber, C.A. (1987) *J. Cell Sci.*, **6** (Suppl.), 97–110.
- Valerie, K., DeRiel, J.K. and Henderson, E.E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7656–7660.
- Valerie, K., Green, A.P., DeRiel, J.K. and Henderson, E.E. (1987) *Cancer Res.*, **47**, 2967–2971.
- Van Duin, M., de Wit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, H.M.H., Hoeijmakers, J.H.J. and Bootsma, D. (1986) *Cell*, **44**, 913–923.
- Van Duin, M., Janssen, J.H., De Wit, J., Hoeijmakers, J.H.J., Thompson, L.H., Bootsma, D. and Westerveld, A. (1988) *Mutat. Res.*, **193**, 123–130.
- Van Houten, B., Gamper, H., Holbrook, S.R., Hearst, J.E. and Sancar, A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 8077–8081.
- Weiss, W.A. and Friedberg, E.C. (1985) *EMBO J.*, **4**, 1575–1582.
- Westerveld, A., Hoeijmakers, J.H.J., van Duin, M., de Wit, J., Odijk, H., Pastink, A. and Bootsma, D. (1984) *Nature*, **310**, 425–429.
- Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1373–1376.
- Wilcox, D.R. and Prakash, L. (1981) *J. Bacteriol.*, **148**, 618–623.
- Yeung, A.T., Mattes, W.B., Oh, E.Y. and Grossman, L. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 6157–6161.

Yeung, A.T., Mattes, W.B. and Grossman, L. (1986) *Nucleic Acids Res.*, **14**, 2567–2582.

Received on June 3, 1988; revised on July 14, 1988

Note added in proof

While this paper was in press, it was agreed to reverse the nomenclature of the first two CHO complementation groups to correspond with the ERCC human repair gene. Hence the group 2 referred to in this paper is now group 1 and vice versa.