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Extrachromosomal recombination occurs efficiently in cells defective in various DNA repair systems

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ABSTRACT

A series of different frameshift mutations of a firefly luciferase reporter plasmid was created so that no activity was obtained when they were transfected into mammalian cells. Co-transfection of these constructs with short fragments of the original sequence resulted in luciferase activity in different cell lines (A-549, NIH 3T3 and Jurkat). The level of this activity was dependent on the length of the fragment, regardless of cell line examined. Two different transfection techniques (lipofection and adenovirus-enhanced gene transfer) gave similar results. It was shown by polymerase chain reaction that expression of detectable luciferase required recombination of the transfected molecules. Cells with defined defects in DNA repair pathways were examined for their ability to perform this extrachromosomal recombination. Cells lacking normal Ku p80, (ADP-ribosyl)transferase, MLH1 or XP-C were all capable of restoring expression to the frameshifted constructs. Given the pivotal roles of the above molecules in the pathways of DNA repair, it seems that this recombination derives from a different activity.

INTRODUCTION

DNA introduced into mammalian somatic cells is acted on by the cellular DNA repair and recombination machinery. Transfected substrates have been used to examine general recombination in a variety of cell types, with selectable marker genes (1–9) or reporter constructs such as *lacZ* (10,11) for quantitation. Mismatch repair has been investigated productively using non-chromosomal DNA (12). Since the luciferase reporter gene can be analysed rapidly and is quantitated easily over a wide range (13), it seemed to be a useful tool for examining extrachromosomal recombination (ECR) in a non-selective assay. We developed a transient transfection assay using short fragments of the luciferase gene to correct non-expressing frameshifts which could be performed in a wide range of cell types. Parameters important in homologous recombination (HR), such as length of the homologous fragment (14,15) or size of the frameshift mutation, could be tested using this assay.

Since ECR has been used in DNA damage-sensitive cell lines to examine the relationship of DNA repair and recombination (3,4,9,11), this assay was performed in cell lines mutant in various DNA repair genes, so that the nucleotide excision, mismatch repair and double-strand break pathways of DNA repair (reviewed in 16,17) could each be examined for their involvement. The CHO-derived line xrs-6, defective in Ku p80, which binds DNA ends and is associated with DNA-dependent protein kinase (18,19), was assayed, as were cells from mice lacking ADPRT (20), an abundant nuclear protein believed to be involved in DNA recombination (21). Other lines investigated were HCT116, a cell line defective in *MLH1* (involved in mismatch repair; 22,23) and GM02249d, derived from an XP-C patient (24,25). An AT line (GM01525e) was also examined, as it had been reported that very high recombination levels could be seen in AT cells, though in an intrachromosomal context; ECR rates were similar to those observed in control lines (10,11). Since all the mutant cells were as capable of carrying out ECR, as measured with the assay we describe, as their respective control lines, we conclude that it is not dependent on any single one of the above enzymes.

MATERIALS AND METHODS

Cell lines

All media and calf sera were from Gibco BRL (Gaithersburg, MD) and were supplemented with 2 mM L-glutamine and antibiotics. NIH 3T3 (mouse fibroblast) and A-549 [human lung carcinoma, American Type Culture Collection (ATCC) CCL 185] were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Primary mouse fibroblasts from both ADPRT knockout mice and wild-type animals were obtained from Dr Z.-Q.Wang (IMP, Vienna, Austria) and were grown in DMEM, 10% FCS with the addition of 5×10^{-5} M β-mercaptoethanol. CHO-K1 and xrs-6 lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and were plated in Ham's F-12 nutrient mix supplemented with 10% FCS. HCT116 lines were obtained from Dr A.Umar (National Institute of Environmental Health Science, NC) and were kept in 1:1 DMEM/Ham's F-12 medium with 10% FCS; HCT116 stably transfected with chromosome 3.6 was kept under selection with 400 μg/ml (active weight) G-418 (Sigma, St Louis, MO). Jurkat E6-1 (human acute T cell leukaemia, ATCC TIB 152) was passaged in RPMI 1640 medium supplemented with 10% FCS. EBV-transformed B lymphoblastoid lines GM02249d (XP-C), GM01525e (AT)

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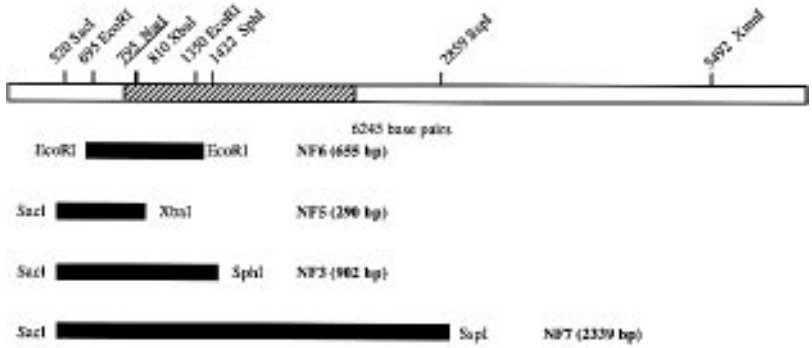

Figure 1. Diagram of pCMV-L showing relevant restriction sites. The region coding for luciferase is shaded and the *Nar*I site around which the frameshifts were constructed is underlined. Fragments NF3, NF5, NF6 and NF7 are shown in black relative to pCMV-L.

and GM00558b were obtained from Coriell Cell Repositories (Camden, NJ) and were grown in RPMI 1640, 15% FCS.

Vector construction

Plasmid pCMV-L (Fig. 1), consisting of the *Photinus pyralis* luciferase gene under control of the CMV promoter, has been described (26). This plasmid was linearised at a unique *Nar*I site in the luciferase sequence and either treated with S1 nuclease for different lengths of time and then filled in using T4 polymerase or simply filled-in using the polymerase before religation. This provided plasmids pNXS-1, pNXS-2 and pNXX, as shown in Table 1. Fragments of pCMV-L containing the *Nar*I site (shown in Fig. 1) were subcloned into the appropriate sites of pUC19 using standard techniques (27). The sequences of all constructs used for this work were confirmed by restriction digestion and DNA sequencing.

Table 1. Frameshift mutations in the luciferase gene used in this work

Plasmid name	Sequence[a]	Frameshift
pCMV-L	aaaggcccggcgccattct	Original sequence
pNXS-1	aaaggcccgg----attct	4 bp deletion
pNXS-2	aaag-------gccattct	7 bp deletion
PNX-X	aaaggcccggc**gc**gccattct	2 bp insertion

[a]Sequence shown is around the unique *Nar*I (ggcgcc) site in pCMV-L.

Plasmid DNA was prepared using column chromatography (Qiagen, Hilden, Germany), following the manufacturer's instructions. The subcloned repair fragments were digested as appropriate and purified from TAE–agarose gels using GeneClean II silica matrix (Bio 101, Vista, CA), following the supplied protocol.

Transient assay for luciferase expression

For all transfections, 3.7 µg frameshifted luciferase reporter plasmid was used as the target. The mass of the fragment DNA used was dependent on its length, as the same molar ratios of target to fragment (1:2, though for the GM lines and primary fibroblasts a ratio of 1:4 was chosen) were used for comparative experiments. Vector DNA (pUC19 or pSP65) was used to ensure that the same absolute amount of DNA was used in each set of experiments; this amount was 6.6 µg for all but the GM lines and the primary fibroblasts, for which it was 9.4 µg. Plasmid DNAs were linearised with *Xmn*I prior to transfection and were heat denatured for 10 min at 95°C just before mixing with the unheated repair fragments, which were then added to the transfection complexes as described below. This heat denaturation did not have a significant effect on expression, though linearisation of the substrates was necessary for good recombination. Luciferase assays were performed as described (13,28). Cells were harvested either 24 or 40 h after transfection and were washed once with phosphate-buffered saline and then harvested in 0.1% Triton X-100, 250 mM Tris–HCl, pH 7.3. Luciferase activity was measured from an aliquot of the supernatant using a Clinilumat LB9502 instrument (Berthold, Bad Wildbad, Germany). Protein content was measured by Bradford dye binding using the BioRad protein assay (BioRad, Hercules, CA); this was performed routinely to avoid intra-assay variation.

Transfection

Adherent cells (NIH 3T3, A-549, HCT116, CHO-K1, xrs-6 and primary mouse fibroblasts, passages 5 and 6) were plated overnight in the appropriate medium at a density of 10^5/well of a 6-well dish (Nunc, Roskilde, Denmark). Jurkat and GM cells were plated at 10^6/well of a 24-well dish (Nunc) 2–4 h before transfection. Transfection conditions appropriate for each of the cell lines used were defined using control reporter constructs.

LipofectAMINE™ was purchased from Gibco BRL. For lipofection of NIH 3T3, A-549, HCT116, CHO-K1, xrs-6 and primary mouse fibroblast cells, DNA was diluted to 50 µl with HEPES-buffered saline (HBS; 20 mM HEPES, pH 7.3, 150 mM NaCl) and added to LipofectAMINE[a] in a final volume of 200 µl, made up with DMEM without serum. The ratio used was 6.6 µg DNA:12 µg lipid. Complexes were allowed to form over 45 min, after which time they were added to cells in a final volume of 1 ml, made up with serum-free DMEM. After 3 h, the medium was replaced with that appropriate for the cell line in question, without selective agents.

Biotinylated adenovirus stock dl1014 (E4 defective; 29) was prepared and inactivated as previously described (30–32). Streptavidin–polylysine (StrApLys) and human transferrin–polylysine (TfpLys) conjugates were prepared according to published methods (28,32) The coupling of OKT3, an α-CD3 antibody for DNA transfer into T lymphocytes, to polylysine (αCD3-pLys) has been described recently (33). Transferrinfection

of A-549 cells proceeded as follows. An aliquot of 10^9 biotinylated virus particles (10^4/cell) was incubated at room temperature with 0.5 µg StrApLys for 30 min in a volume of 150 µl HBS, after which the DNA was added in a volume of 100 µl. After 30 min, 2.5 µg TfpLys was added, to a final volume of 300 µl. Transfection complexes were allowed to form for 30 min, then were added to the cells in a final volume of 1 ml, made up with the appropriate medium. Jurkat cells were transfected in essentially the same manner, save that 1.0 µg StrApLys was used and the TfpLys was replaced with 4 µg αCD3–pLys. For transfection of the GM lines, volumes were as above, but 5×10^9 particles (5×10^3/cell) were used to transfect 9.4 µg substrate DNA, along with 2 µg StrApLys and 2.5 µg TfpLys/7.5 µg pLys in place of the TfpLys alone. Transfection complexes were left on the cells for 3 h, after which time the medium was replaced.

PCR

DNA was phenol/chloroform extracted from cell lysates of NIH 3T3 cells transiently transfected with pNXS-2 and NF3 correcting fragment. This lysate was positive for luciferase. After ethanol precipitation into Tris–EDTA buffer, oligonucleotide sequences appropriate for discrimination between frameshifted and repaired plasmid and repair fragment were used to amplify the DNA, as shown diagrammatically in Figure 4A. These were (1) 5′-GGCAG-TACATCAATGGG-3′, lying outside the *Sac*I–*Sph*I fragment and (2) 5′-GATAGAATGGCGCCGGGC-3′, the 3′-end of which lay in the deleted sequence. A mixture of pNXS-2 and NF3 was used as negative control and pCMV-L was used as a positive control for PCR (34). To ensure that sufficient target was present for amplification, the primers (3) 5′-GCTTGGGAATTCCTTTGTGT-TACA-3′ and (4) 5′-GAAGAGAGTTTTCACTGC-3′ were used to amplify a region of the luciferase gene present in all samples.

RESULTS

Characteristics of this ECR assay

Somatic cells are capable of performing homologous recombination on transfected substrates. The luciferase reporter gene appeared to be a promising candidate for studying such recombination. Different plasmids containing short frameshifting deletions in the reporter gene (as shown in Table 1) were transfected into A-549 and NIH 3T3 cells. As shown in Figure 2, no luciferase activity was detected after transfection. However, expression could be restored to all the constructs tested in both cell lines by co-transfecting a small, non-expressing fragment of the original sequence (Fig. 2). Efficiency appeared to be independent of the size of the frameshift, within the range tested here, though the ease with which the cell line could be transfected was important.

Chromosomal assays using HR to target various genes have shown the importance of homology length in recombination efficiency (14,15). To assess whether this dependence was present in this system, equimolar amounts of differently sized repair fragments were prepared and co-transfected with pNXS-1 into different cell lines as described. Restored expression was highly dependent on the length of the fragment (Fig. 3a and b). Cells transfected with NF5, NF3 or NF6 in the absence of plasmid target gave a basal level of luciferase activity, as in untransfected cells. Although NF7 transfected into NIH 3T3 cells gave a

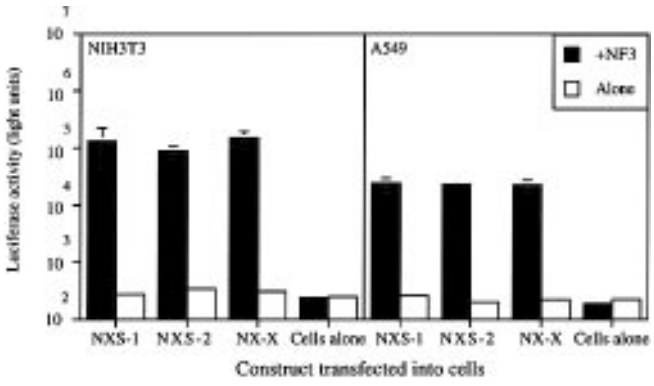

Figure 2. Ability of cells to correct different frameshift mutations. NIH 3T3 and A-549 cells were transfected by lipofection with 3.7 µg of the indicated plasmid, with or without 1.1 µg NF3 fragment. Samples were analysed at 24 h. The means ± SD shown are from at least two separate experiments. Total light units per well are shown.

relatively high background, this was still 100- to 1000-fold lower than the activity after co-transfection with pNXS-1. In all other cells, transfection with NF7 alone resulted in no luciferase expression. Luciferase activity with all other constructs was the same as in untransfected cells. This size dependence was observed in NIH 3T3, A-549 and Jurkat cells. While the absolute values varied from cell line to cell line, the length dependence of luciferase expression was reproducible. Linearisation of the substrates in such assays has been shown to be important (2,35). Co-transfection of undigested frameshifted plasmids with circular vectors carrying the fragments could generate expression, but this was 5- to 10-fold lower than that obtained with the linear substrates (data not shown).

Since two transfection systems, namely adenovirus-enhanced ligand-mediated transfection and lipofection, were to be used in subsequent experiments, both were tested in A-549 cells. Despite slightly differing transfection efficiencies, the dependence of expression on the length of the repair fragment was clearly shown (Fig. 3a). We concluded that none of the effects seen were due to the method of transfection used. Time course experiments showed only a slight increase in luciferase activity between 24 and 40 h (data not shown), so that time of harvesting does not influence the results presented here.

PCR was used to show whether the frameshift had been corrected in cells expressing luciferase (Fig. 4). With the 5′-end of one primer lying in the 7 bp deletion of pNXS-2 and the other primer lying outside the repair fragment on the luciferase sequence, an *in vitro* mixture of substrates gave no product (Fig. 4B, upper panel, lane ii). After the recombination necessary for expression, such amplification could be detected (Fig. 4B upper panel, lane i). Non-discriminatory primers were used to show that sufficient target was present in each case for amplification (Fig. 4B, lower panel).

Ability of cells defective in DNA repair to restore expression to frameshifted plasmids

In order to examine whether mutations and defects in the pathways of DNA repair have any effects in this ECR assay, we examined primary ADPRT$^{-/-}$ mouse fibroblasts, the X-ray-sensitive CHO line xrs-6, defective in Ku p80, the hMLH1-defective line

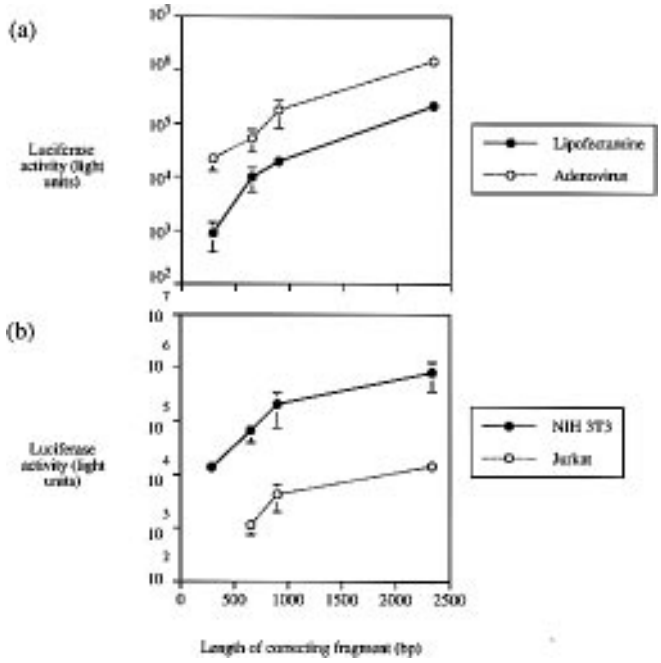

Figure 3. Dependence of restored expression from pNXS-1 on the length of co-transfected repair fragments using different transfection methods and in different cell lines. (**a**) Results shown were obtained with transfections carried out using lipofection and adenovirus-enhanced transferrinfection in A-549 cells. (**b**) Results were obtained using lipofection in NIH 3T3 and adenovirus-enhanced gene transfer in Jurkat cells, as described in Materials and Methods. Data were obtained 40 h after transfection and are the means ± SD of at least three separate experiments. Total light units per well are shown. Data shown have had any background subtracted. Average light units per well in control experiments with 3.7 µg intact pCMV-L were 6×10^7 (A-549 cells, adenovirus-enhanced transferrinfection), 3×10^7 (A-549, lipofection), 3.5×10^7 (NIH 3T3) and 6×10^6 (Jurkat) light units per well, using the conditions described.

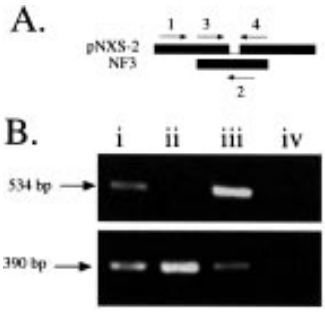

Figure 4. PCR analysis of pNXS-2 co-transfected with NF3 into NIH 3T3 cells. The location of the primers is as shown in (**A**). The upper panel of (**B**) shows amplification using primers 1 and 2, while the lower shows results using 3 and 4. The targets for amplification are: (i) transfected cells with restored expression; (ii) pNXS-2 and repair fragment mixed *in vitro*; (iii) pCMV-L; (iv) water blank.

HCT116, the XP-C line GM02249d and the AT line GM01525e. pNXS1 and the NF3 fragment were transfected into these mutant cell lines and, where possible, into similar, non-mutant cell lines. These lines were CHO-K1, the parental line for xrs-6 (3,4,18,19), HCT116 stably cloned with human chromosome 3.6, which complements its mismatch repair mutation in an *in vitro* assay

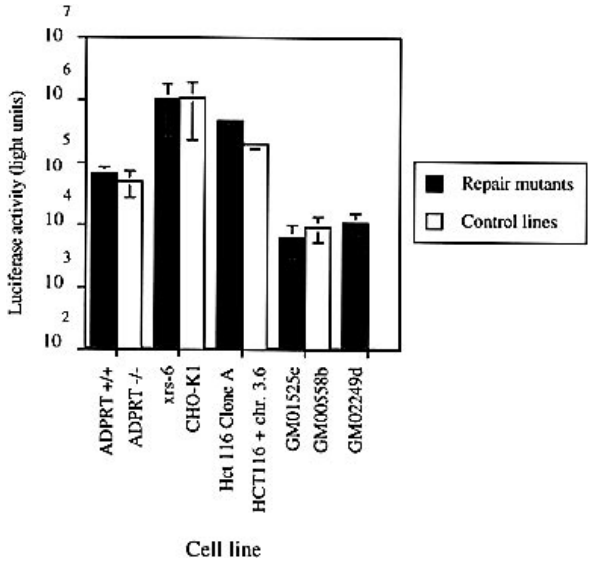

Figure 5. Ability of mutant cell lines to correct frameshifts in a luciferase plasmid. Cells were transfected with pNXS-1 and NF3 as described in Materials and Methods. The GM lines were not transfected with the same efficiency, so that the values presented here have been corrected using their positive controls, in order to compare them. Results given are total light units per well and show the mean ± SD from at least two separate experiments. Control experiments using the conditions described to transfect intact pCMV-L gave average values of 3×10^6 light units in the GM lines, 6×10^7 in the xrs-6 and CHO-K1 lines, 2.5×10^7 in the mouse primary fibroblasts and 5×10^7 in the HCT116 lines.

(36), wild-type mouse cells for the ADPRT knockouts and a line from an apparently normal donor, GM00558b, for the lymphoblastoid cells. All the mutant cell lines could effectively correct the frameshifts, as comparisons of xrs-6 and CHO-K1, of HCT116 and HCT116 + chromosome 3.6 and of ADPRT$^{-/-}$ fibroblasts with wild-type cells showed no significant differences in expression (Fig. 5). Expression was restored in the XP-C line GM2249d and in the AT line GM01525e as efficiently as in the control lymphoblastoid cell line GM00558b. Dependence of restored luciferase expression on the size of the correcting fragment, as had been observed for NIH 3T3, A-549 and Jurkat cells (Fig. 3), was observed in all these lines (data not shown).

The absolute values of restored expression were dependent on the ease with which the lines could be transfected, which was dependent on each cell type and the method used. The expression vector pCMV-L was used as a positive control in each set of experiments, so that an idea of the relative transfection efficiencies with the differing cell types and transfection protocols could be gained. Values for such transfections were 100- to 1000-fold higher than those obtained with correction of the frameshifted plasmid (data not shown).

DISCUSSION

Our results show that recombination between a frameshifted luciferase reporter plasmid and short DNA fragments containing the original sequence occurs efficiently in mammalian somatic cells and results in restoration of luciferase expression. Using the transfection systems available in our laboratory, such recombination could be observed in a number of cell lines, including some normally refractory to transfection (Jurkat and lymphoblastoid

cells). This restoration of expression can be observed with frameshifts of different sizes and is dependent on the length of the DNA fragment used to achieve this restored expression. The strong length dependence observed here compares closely with results on HR using selective markers (14,15), though the lengths of the constructs are rather shorter due to the limit imposed by the luciferase gene.

This assay is not selective, so that individual clones could not be examined for the structure of the molecules involved in restored reporter expression. However, PCR analysis showed that recombination of the substrates is necessary for the generation of restored reporter gene expression (Fig. 4). After luciferase expression in NIH 3T3 cells was regenerated with these DNAs, a product could be amplified with the discriminatory primers, indicating that they were now on the same, recombined molecule responsible for expression of the reporter gene. Where the 5′ end of one primer lay in the 7 bp deletion of pNXS-2 and the other primer lay outside the repair fragment on the luciferase sequence, an *in vitro* mixture of substrates gave no amplification, indicating that expression is due to replacement of the frameshift by the correct sequence.

We then investigated what activities were responsible for this. It has been observed that double-strand breaks are recombinogenic (6,35,37). The introduction of the substrate DNAs described here might be seen as the introduction of a large number of double-strand breaks. Recent work has shown that Ku protein binds to DNA breaks and that its deficiency, as in xrs-6 cells, leads to defects in double-strand break repair and V(D)J recombination (18,19,38,39). The absence of any significant difference between control (CHO-K1) and xrs-6 cells indicated that Ku can be omitted from this system without hampering ECR, which had not been wholly clear from previous evidence (3,4). Another cellular component which binds to DNA breaks is ADPRT (21,40). The availability of mouse cells lacking this enzyme (20) made possible an examination of whether there was a crucial role for this enzyme in the recognition of the substrates used in this assay. As shown in Figure 5, this was not the case; the kinetics of restoration of expression were the same in the ADPRT$^{-/-}$ cells and the controls (data not shown). The recognition of the substrates here as double-strand breaks, if involved in this correction, is not reliant on Ku or ADPRT.

If the restoration of expression to the frameshifts used in our system generates substrates analogous to the insertion/deletion mutants used for the examination of mismatch repair mutants, restoration of luciferase expression should be hindered in cells defective in mismatch repair. The DNA repair systems involved in mismatch correction, which have been linked to HNPCC in humans, are also capable of repairing small insertion/deletion mismatches (16,23). These mismatches consist of small (≤5 bp) bulges in DNA, due to a region of unpaired bases in the duplex, and can lead to large variations in the structure of the DNA (41); p53 is implicated in their recognition (42). The absence of the mismatch repair protein hMLH1 from extracts of HCT116 cells has been shown to prevent their repair of DNAs containing small (≤5 bp) bulges (23). However, efficient expression of luciferase was detected in these cells, indicating correction of the frameshift and that the substrates described need not necessarily be repaired by the mismatch repair system. The lack of effect of differing frameshifts, as shown in Figure 2, is in keeping with this finding, and shows that ECR is essentially independent of mismatch repair, though this is not the case in chromosomal HR (43).

The lesions recognised by the nucleotide excision repair system (pyrimidine dimers and chemical adducts) are rather different from those provided in our assay (24). The ability of XP-C to recombine transfected DNA was not impeded by the mutation which contributes to their DNA damage sensitivity, though this might have been expected in XP-C cells, which have a repair activity on transcriptionally active DNA (24,25,44). This suggests that this pathway is not involved in restoration of expression by ECR. A similar conclusion may also be drawn for the mutation in AT, which disease involves problems in DNA metabolism.

The provision of so many DNA ends to the cells in our assay may overwhelm the specific systems of DNA repair, so that even if the mutations have an effect, they may not be seen. Despite this caveat, these results point toward the existence of a non-specific system acting to recombine transfected DNA, the action of which is not dependent on the molecules whose mutants were tested here.

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