The rejoining of double-strand breaks in DNA by human cell extracts

Phillip North, Anil Ganesh and John Thacker*
MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 0RD, UK

Received September 4, 1990; Revised and Accepted October 9, 1990

ABSTRACT

A double-strand DNA break was introduced at a specific site within the *lacZ* gene of plasmid pUC18 using one of several restriction enzymes, and the plasmid exposed to nuclear extracts from human cell lines. Physical rejoining of DNA was monitored by Southern analysis after gel separation, and the fidelity of rejoining by expression of the *lacZ* gene after bacterial transformation with the treated plasmid. Breaks at the *Sal*I and *Eco*RI sites were rejoined by extracts to form circular monomers, but the efficiency of rejoining was much higher at the *Sal*I site. Measurement of rejoining at several adjacent sites having different types of termini, consistently showed a range of efficiencies with 5′ 4-base > 3′ 4-base overhangs and 4-base > 2-base > no overhang. Similar efficiencies were found for nuclear extracts from transformed cell lines, both from a 'normal' individual and an ataxia-telangiectasia (A-T) patient, and from a non-transformed normal cell culture. In contrast at some sites, especially those with a low rejoin efficiency, the *fidelity* of rejoining was very much lower for the A-T extracts than for normal cell extracts. Mis-rejoining was, however, unrelated to rejoin efficiency at other sites, suggesting that factors such as the exact sequence at the break site on the molecule may also influence the fidelity of rejoining.

INTRODUCTION

The introduction of defined and localized sites of damage into purified DNA molecules, followed by exposure of the DNA to a cellular environment, is an important technique in the molecular analysis of DNA repair and mutagenesis in mammalian cells (e.g., 1–5). Localization of a damage site allows it to be followed thereafter for mechanistic analysis of subsequent processing.

An important type of DNA damage, induced by agents such as ionising radiation, is the double-strand break (dsb). A number of studies implicate the dsb as a lethal lesion in cells (6–8), and some radiation-sensitive mutants of mammalian cells appear to have a reduced ability to rejoin this type of damage (9–11). Individuals with the disorder ataxia-telangiectasia (A-T), who suffer both radiosensitivity and cancer-proneness (12–14), may also be unable to repair DNA breaks adequately in comparison to normal human cells (15,16).

In the present study we have attempted to refine the measurement and analysis of DNA-break rejoining with the use of nuclear extracts from human cells applied to defined molecules carrying specific enzymatically-induced dsb. As prototypes we have compared the activities of extracts from an A-T cell line (AT5BIVA) with those from a line showing normal radiation-sensitivity (MRC5V1). The simple recombinant plasmid pUC18 was used as a substrate because it has a number of different enzyme break sites closely spaced at the same location (the multicloning site) on the molecule. This site is within the *lacZ* gene of pUC18, allowing the fidelity of rejoining to be assessed by expression of normal gene activity after extract treatment.

MATERIALS AND METHODS

Cells and extracts

The transformed human cell lines MRC5V1 (17) and AT5BIVA (18) and the non-transformed HF12 cells (19) were grown in Eagle's Minimal Essential Medium (MEM), supplemented with 1 mM glutamine, 10% foetal calf serum and antibiotics, at 37°C in 5% CO_2:95% air. The sensitivity of these cell lines to ionising radiation was checked by colony-forming assay; AT5BIVA was approximately 4-fold more sensitive to X-rays than MRC5V1 or HF12 (data not shown), in agreement with previous data (12,13).

Cells were held as frozen stocks until required for extract preparation; they were grown up to final numbers in excess of 10^8 cells using large numbers of flasks or petri dishes. Extract procedures were based on those of Lopez et al. (20); ice-cold conditions were used throughout. Cells were scraped into phosphate-bufferred saline (PBS) using a 'rubber policeman' and washed 3 times in PBS with centrifugation. The cell pellet was resuspended in 10 ml buffer A (Tris-HCl, pH 7.5, 20 mM; $MgCl_2$, 0.5 mM; dithiothreitol, 0.5 mM; KCl, 0.5 mM; $CaCl_2$, 2 mM) containing 250 mM sucrose, and held for 15 min. After centrifugation, the cells were resuspended in buffer A without sucrose but with PMSF (phenylmethylsulphonyl fluoride, 1 mM) and homogenized (40 strokes) in a Dounce homogeniser to release intact nuclei. Nuclei were pelletted (2000 g, 1 min) and washed 4 times in buffer A with triton X-100 (0.5%) and 3 times without triton but with PMSF. Nuclei were resuspended in 2 ml buffer B (NaCl, 500 mM; EDTA, 10 mM; PMSF, 0.1 mM) and sonicated for 4×15 sec bursts. Debris was pelletted in microtubes

* To whom correspondence should be addressed

(12000 rpm for 30 min). Protein was precipitated from the supernatant by adding ammonium sulphate (0.34 g/ml) and stirring for 2 hrs. The precipitate was pelletted at 15000 g for 20 min; then it was dissolved and dialysed overnight in buffer C (Tris-HCl, pH7.5, 50 mM; EDTA, 0.1 mM; 2-mercaptoethanol, 10 mM; PMSF, 0.1 mM; glycerol, 10%) before use or storage at −70°C for up to 6 months. Protein concentration in the extracts was measured using the Biorad protein assay.

Plasmid preparation

HB101 bacteria containing the plasmid pUC18 were grown in bulk culture, and plasmid DNA was isolated and purified using standard methods (21). Double-strand breaks were introduced at single sites in the multicloning region of pUC18, to interrupt the *lacZ* gene, using restriction endonucleases (Bethesda Research Labs) as shown below.

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GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTC
   HindIII      PstI ------      BamHI          ------ EcoRI
                    SalI/                        SstI
                AccI/HincII
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The linearized plasmid was phenol/chloroform purified; breakage was checked by agarose gel electrophoresis and Southern analysis.

Reaction conditions

Plasmid DNA and protein extracts were mixed in 50 μl reactions containing Tris.HCl (pH7.5, 65.5 mM), $MgSO_4$ (10 mM), ATP (1 mM),EDTA (91 nM), 2-mercaptoethanol (9.1mM), PMSF (91 nM), glycerol (9.1%), and DNA (40 μg/ml). Nuclear-extract protein (0.2−3.8 mg/ml) or T4 ligase (BRL; 0.01 U) was added to start the reaction. Incubation was at 14°C for 20−26 hrs. DNA was subsequently purified by phenol/chloroform extraction and ethanol precipitation. Some reactions were flash frozen in liquid nitrogen and stored at −70°C before purification.

Gel electrophoresis and Southern analysis

After a brief heating (65° for 10 min) a quarter of the sample was run on a 1% agarose gel to estimate the DNA concentration. Subsequently 1 ng of DNA from each sample was run on a 1% agarose gel (without ethidium bromide, unless otherwise noted) in TBE buffer (21), blotted on to nitrocellulose (Schleicher and Schull), and probed with pUC18 labelled by the random oligonucleotide method (22) to a specific activity of $0.4-2.0\times10^9$ cpm/μg DNA.

Bacterial transformation and rejoin fidelity

Bacterial transformations were carried out using the *E. coli* strain DH5alpha (23) and the standard transformation protocol of Hanahan (24). The transformants were selected on LM plates containing ampicillin (100 μg/ml) and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside:40 μg/ml). Bacterial viability was assessed on plates containing X-gal but no ampicillin. White colonies (not expressing β-galactosidase activity) were streaked onto LM plates with ampicillin and X-gal for confirmation. Transformation frequencies were expressed as transformants per viable cell to allow comparison between different experimental samples.

Analysis of mis-rejoined plasmid DNA

White colonies, previously streaked onto LM plates, were used to inoculate cultures in nutrient broth with ampicillin. After 12−14 hr growth the bacteria were harvested and plasmid isolated using the alkaline-lysis method (21). After exposure to various restriction enzymes (see text) the DNA was run on either 1% agarose or 5% polyacrylamide gels.

RESULTS

Conditions for break rejoining by human nuclear extracts

Extracts prepared from nuclei of the radiation-sensitive cell line AT5BIVA and its normal counterpart MRC5V1 were found to be able to rejoin broken plasmid molecules to form both circular monomers and linear catenates. A typical experiment showing the untreated, broken and extract-treated plasmid DNA is given in Fig.1 to illustrate the formation of rejoined molecules and the controls. The enzyme T4 ligase was used as a positive control in experiments, while nuclear extracts were boiled as negative controls. It is seen that unbroken DNA has predominantly closed circular monomers (CC) although some open circular (OC) and dimeric forms are present; when broken with endonuclease a single linear (LIN) band is found. Rejoining with extract or T4 ligase yields a set of CC-forms (topoisomers, seen when gels are run without ethidium bromide present) and linear multimers, while boiled extract does not. The bacterial transformation frequencies found with these DNA samples are given in the legend to Fig.1; it was found that measured transformation frequencies and crude visual estimates of the amount of rejoined circular monomers on gel blots were in general quantitative agreement. As expected, breaking the DNA reduced the

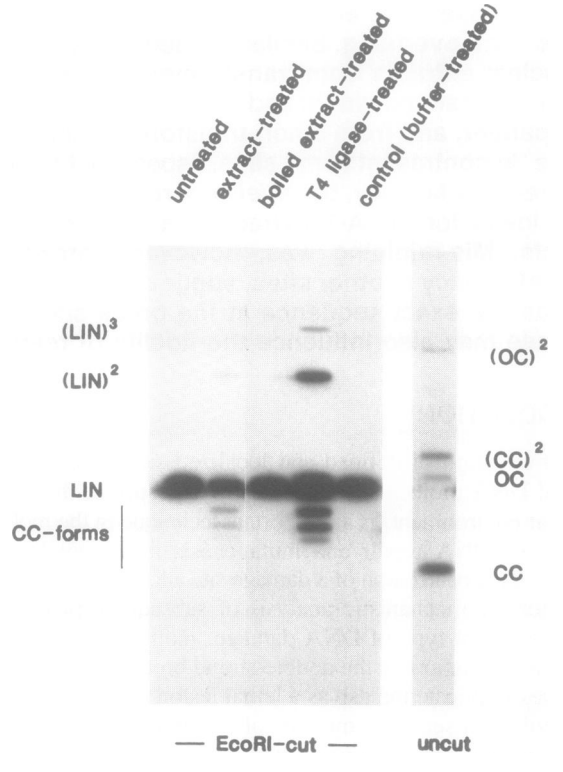

Fig. 1. Southern blot hybridization to unbroken or *Eco*RI-cut (treated and untreated) pUC18 DNA after electrophoretic separation. The extract protein concentration was 0.6 mg/ml. DNA forms: CC, closed circular; OC, open circular; LIN, linear (also multimeric forms shown by powers). These samples yielded bacterial transformation frequencies per 10^6 viable cells as follows: uncut 392; cut/untreated 1.1; cut/extract treated 4.3; cut/boiled-extract treated 1.1; cut/T4-ligase treated 277.

transformation frequency by about 400-fold (25,26), while extract (and T4 ligase) treatment of broken DNA increased this frequency significantly.

The influence of reaction conditions was explored as follows: (a) protein concentration: the production of CC monomers and other forms was concentration dependent, but saturated at different concentrations depending on the site of the break. This is illustrated in Fig.2 for pUC18 broken at the *Eco*RI or *Sal*I sites (see also below).
(b) plasmid DNA concentration: preliminary experiments made at a j:i ratio (27) favouring the formation of circular monomers yielded few rejoined molecules, while the relatively high concentration of 40 μg/ml gave consistent formation of circular monomers and linear dimers.
(c) cofactors: the formation of circular monomers required ATP and magnesium ions, but not dNTPs (data not shown). A long incubation time was allowed for maximum formation of rejoined molecules, although we have found that this can be shortened substantially without loss of circular monomer formation.

Rejoining of broken plasmid by normal and A-T nuclear extracts

Extensive data were obtained for pUC18 DNA broken at the *Eco*RI or *Sal*I sites. Fig.3 gives an example of Southern blot data for rejoining at these sites by extracts from nuclei of A-T and normal cells. It is seen that extracts from the different cell types gave approximately the same amount of rejoined material and very similar transformation frequencies (Fig.3 legend). It should be noted, however, that *Eco*RI-broken DNA was rejoined with lower efficiency than *Sal*I-broken DNA by the nuclear extracts (longer exposure of the gel blot in Fig.3 showed the formation of circular monomers with *Eco*RI-broken DNA). Combined data from several experiments gave the average transformation frequencies shown in Table 1; overall these showed a difference of about 5-fold between *Eco*RI-broken and *Sal*I-broken DNA frequencies after rejoining with extracts from either cell type. This difference between the two break-sites was also reflected in the protein-concentration dependence of monomer and multimer formation (Fig.2). In contrast, under these reaction conditions, rejoining by T4 ligase was more efficient at the *Eco*RI site than at the *Sal*I site (data not shown).

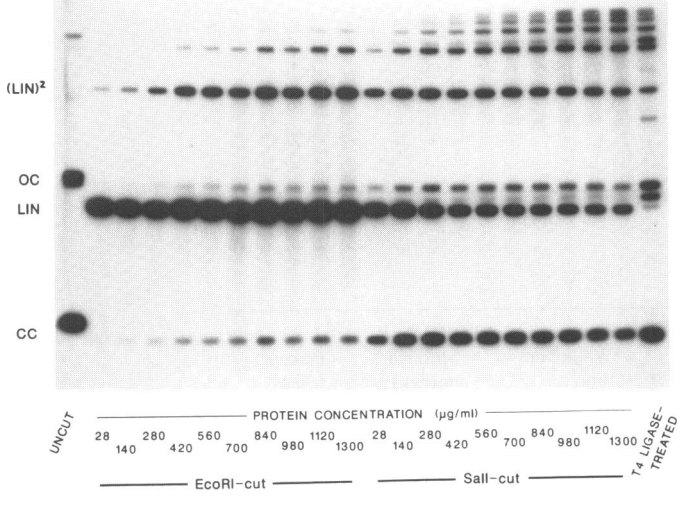

Fig. 2. Effect of increasing AT5BIVA nuclear extract protein concentrations on the rejoining of pUC18 broken at the *Eco*RI or *Sal*I sites. Gel run in the presence of ethidium bromide, to consolidate the CC forms in one position; note that this position is slightly different from that for the original (bacterial) uncut material.

Fidelity of rejoining of endonuclease-broken plasmid

The fraction of pUC18 plasmids yielding white colonies, by mis-rejoining of the broken *lacZ* gene, was measured in control samples consisting of unbroken DNA, untreated broken DNA, or broken DNA exposed to boiled extracts or T4 DNA ligase. It is seen (Table 2) that this fraction is extremely low in unbroken DNA, while untreated broken DNA gives a higher white colony fraction presumably due to the action of the bacterial host cells on the DNA ends (see Discussion). DNA treated with boiled nuclear extracts is 'mis-rejoined' to a similar extent to untreated broken DNA; a small increase may occur with *Eco*RI-broken DNA, but the numbers counted are relatively few. Treatment with T4 ligase reduces the mis-rejoined fraction in broken DNA by approximately 10-fold at either break-site.

The results of large-scale transformation experiments to give measurable frequencies of white colonies from extract-treated DNA are shown in Tables 3 and 4, for *Eco*RI-broken and *Sal*I-broken DNA respectively. The fraction of mis-rejoined plasmids found for *Eco*RI-broken DNA exposed to extracts from MRC5V1 cells is relatively similar to that for untreated broken DNA (compare Tables 2 and 3), but this fraction is very high after exposure to AT5BIVA nuclear extracts. This result was

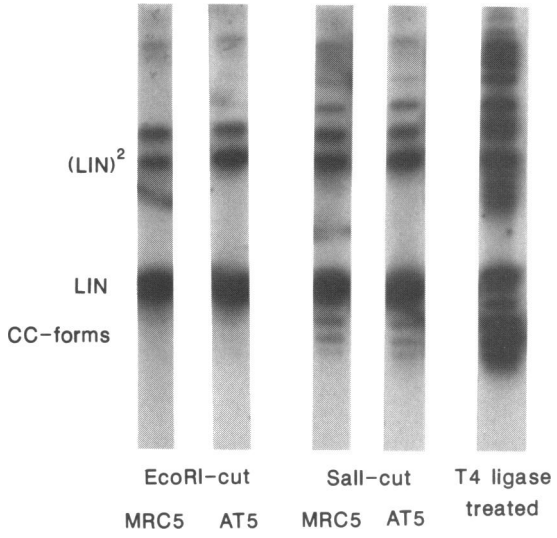

Fig. 3. Southern blot hybridization to broken pUC18 DNA after treatment with different cell extracts at the same protein concentration (0.6 mg/ml) or T4 ligase. DNA forms are indicated as in Fig.1. Bacterial transformation frequencies per 10^6 viable cells were as follows: uncut 108; *Eco*RI-cut: /untreated 1.7, /MRC5-extract treated 4.2, /AT5-extract treated 4.2; *Sal*I-cut: /MRC5-extract treated 25.8, /AT5-extract treated 33.2.

Table 1. Transformation frequencies for DNA treated extracts from human cell nuclei

Cell type	Frequency per 10^5 viable bacteria[a]		
	*Eco*RI-break	*Sal*I-break	Ratio
MRC5V1	1.5 (7)	9.5 (3)	0.16
AT5BIVA	1.9 (16)	8.0 (4)	0.23

[a]Numbers of expts. shown in parentheses; these extracts had a range of protein concentrations, but similar data were found for comparisons at the same protein concentration.

reproducible within a factor of about 2 for independent extracts and experiments (Table 3). In addition, several samples of DNA broken with *Eco*RI were used, and these were put through more stringent purification procedures prior to extract treatment, to ensure that these results did not arise from some spurious effect peculiar to the DNA sample (note also that the rejoining of the *Eco*RI-broken DNA by T4 ligase was not compromised, indicating that poor fidelity did not arise from repair of DNA damaged in the initial production of breaks by the endonuclease). Thus it appears that the A-T nuclear extract lacks fidelity in rejoining these simple breaks.

For the rejoining of *Sal*I-broken DNA (Table 4) a different picture was found; both MRC5V1 and AT5BIVA extract-treatments gave similarly low fractions of white colonies. Since, for a given protein concentration, rejoining at the *Sal*I site is much more efficient than for the *Eco*RI site (Fig.2) we also measured the mis-rejoined fraction for *Sal*I-broken DNA at very low protein concentrations (giving a transformation frequency reduction of 10 or more; i.e., similar to the frequencies found for *Eco*RI-broken DNA). However, the fraction of white colonies was not increased above that found at the higher protein concentrations tested (data not shown).

Table 2. Mis-rejoining in control DNA samples

Treatment of broken DNA	White:blue ratio[1] *Eco*RI-break	*Sal*I-break	Percent whites *Eco*RI-break	*Sal*I-break
Untreated	6: 2873 (5)	7:1546 (7)	0.21	0.45
Boiled extract	41: 5796 (11)	3: 579 (2)	0.70	0.52
T4 ligase	6:15089 (8)	1:1581 (2)	0.04	0.06
[Unbroken[2]	2:35243 (18)			0.005]

[1]number of expts. shown in parentheses
[2]unbroken DNA treated with nuclear extracts also gave a very low fraction of white colonies

Rejoin efficiency and fidelity of AT5BIVA extracts at other break sites

Rejoin efficiency may be affected by degree of 'cohesiveness' of the DNA ends, including both the type and number of bases involved, although it is not clear whether this also affects the rejoin fidelity. Therefore, we examined the efficiency and fidelity of rejoining by AT5BIVA nuclear extracts at several other sites in the pUC18 multicloning region (see Methods). Some of these sites, like *Eco*RI and *Sal*I, generate 5′ 4-base overhangs (*Bam*HI, *Hin*dIII), while others give 3′ 4-base overhangs (*Pst*I, *Sst*I). In addition, we tested *Hin*cII which yields non-cohesive ends, and *Acc*I which breaks the pUC18 DNA at the same site as *Sal*I but has a 5′ 2-base overhang.

As yet, with *Hin*cII-broken DNA we have seen no increase in transformation frequency over control (broken untreated DNA) levels after treatment with any extract and very little formation of circular monomers (although some linear dimers; data not shown). Table 5 shows representative transformation frequencies for DNA broken at each of the other sites, and the proportion of mis-rejoined molecules found. It is seen that *Acc*I-broken DNA is rejoined with relatively low efficiency, even when compared to *Eco*RI-broken DNA, and has a high fraction of mis-rejoined plasmids with AT5BIVA extract treatment. The 5′ 4-base

Table 3. *Eco*RI-break mis-rejoining by human nuclear extracts

Extract no.	Expt no.	MRC5V1 White:blue ratio	(protein mg/ml)	Percent whites	AT5BIVA White:blue ratio	(protein mg/ml)	Percent whites
1	1	4: 874	(0.2)	0.45	557:4948	(0.6)	10.1
					620:4928	(0.9)	11.2
					368:3284	(1.7)	10.1
					70:1014	(2.5)	6.5
	2	3: 877	(0.2)	0.34	662:5868	(3.8)	10.1
	3				678:4221	(0.6)	13.8
	4				544:3501	(3.8)	11.2
2	5	3:1187	(1.7)	0.25	130:2271	(0.6)	5.4
					15: 254	(1.3)	5.6
3	6	9:2251	(0.6)	0.40	115:1653	(0.6)	6.5
					98:1505	(0.9)	6.1
	7	7:3901	(0.6)	0.18			
	8	6:2199	(0.6)	0.27			
	9	0: 169[1]	(0.6)	<0.59			
	10	1: 531	(0.6)	0.25			

[1]DNA concentration lower than in other treatments (j:i >2)

Table 4. *Sal*I-break mis-rejoining by human nuclear extracts

Extract no.	Expt no.	MRC5V1 White:blue ratio	(protein mg/ml)	Percent whites	AT5BIVA White:blue ratio	(protein mg/ml)	Percent whites
1	1	0:1508	(0.2)	<0.07	1:3557	(0.6)	0.03
					5:2822	(3.7)	0.18
	2				0:1787	(3.7)	<0.06
2	3	0: 214	(1.7)	<0.46	1:1271	(0.6)	0.08
					2:1289	(1.3)	0.15
3	4	0:2097	(0.4)	<0.05	0:1792	(0.6)	<0.06
		3:1333	(0.6)	0.22	0: 744	(0.9)	<0.13
	5	1:1805	(0.6)	0.06			

overhang sites (*Bam*HI, *Hin*dIII) have relatively high rejoin efficiencies and a much smaller mis-rejoined fraction than *Eco*RI-broken DNA (Table 5), although this fraction is considerably higher than in controls (Table 2) or *Sal*I-broken DNA (Tables 4 and 5). The 3′ 4-base overhangs have a somewhat lower rejoin efficiency than the 5′ 4-base overhangs, but these two sites show a striking difference in the fidelity of rejoin (percent whites, Table 5). Thus, *Pst*I-broken DNA gave a relatively high mis-rejoined fraction while *Sst*I-broken DNA showed a mis-rejoined fraction only slightly higher than that found for *Sal*I-broken DNA.

Table 5. Transformation frequency and mis-rejoined fractions of pUC18 treated with AT5BIVA extracts after breakage at different sites

Enzyme	Overhang sequence	Transformation frequency[1]	White:blue ratio[2]	Percent whites
*Eco*RI	5′ AATT	1.3	358:5683	5.9
*Sal*I	5′ TCGA	20	3:5096	0.06
*Bam*HI	5′ GATC	9.2	47:3688	1.3
*Hin*dIII	5′ AGCT	13	58:5012	1.1
*Pst*I	3′ TGCA	3.2	98:5102	1.9
*Sst*I	3′ AGCT	5.6	9:4139	0.22
*Acc*I	5′ CG	0.21	29:560	4.9

[1]Values given per 10^5 viable bacteria, all from the same expt. to ensure comparability.
[2]Using extracts 2 and 3, with protein concentrations in the range 0.6– 1.3 mg/ml.

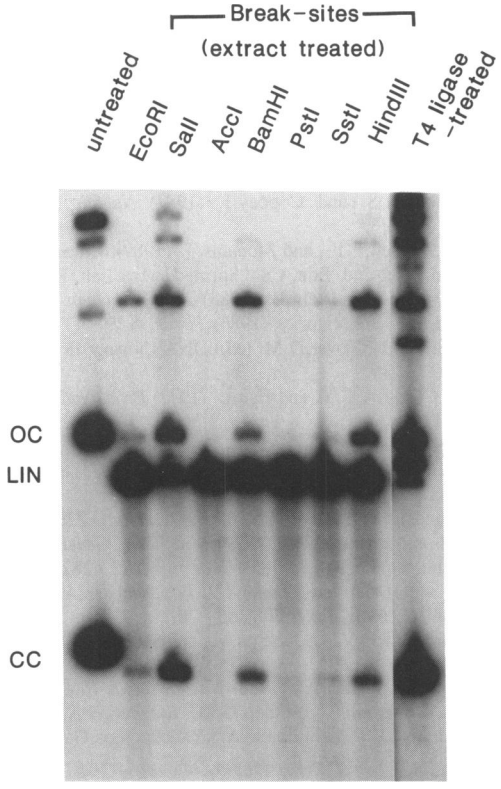

Fig. 4. Efficiency of rejoining of different break-sites in pUC18 by extracts from untransformed HF12 cells (at 0.72 mg/ml protein), compared to unbroken or T4 ligase-rejoined material (gel run with ethidium bromide). Transformation frequencies per 10^5 viable bacteria were: uncut (untreated) 294, *Eco*RI 7.4, *Sal*I 62, *Acc*I 0.8, *Bam*HI 12.5, *Pst*I 1.5, *Sst*I 2.6, *Hin*dIII 29.

Rejoin efficiency of non-transformed cell extracts

The nuclear extracts used for the above assay were isolated from transformed cell lines, which may be atypical of normal (diploid) cells isolated freshly from tissue. However, early passage human fibroblasts were found to show similar relative rejoin efficiencies as those established for the transformed lines over a range of break-sites (Fig.4). Mis-rejoined fractions were relatively low at all break-sites (data not shown).

Analysis of mis-rejoined plasmid molecules

When pUC18 is broken with *Pvu*II a fragment of 322 base pairs, including the whole multicloning site, is released. Plasmid isolated from individual white bacterial colonies gave a range of *Pvu*II fragment sizes, with the majority showing losses of < 100 base pairs, for both MRC5V1 and AT5BIVA treatments. Plasmids which cut once or did not cut with *Pvu*II were analysed on agarose gels using larger fragments generated by *Bgl*I or *Sca*I digests. Fig.5 shows the overall distribution of deletion sizes for 153 plasmids isolated (13 MRC5V1-, 140 AT5BIVA-extract treated).

DISCUSSION

Rejoin efficiency

We have been able to measure successfully the rejoining of 'model' DNA double-strand breaks by human nuclear extracts, under simple reaction conditions. The rejoining appears to be taken to completion by the extracts; it should be noted that the DNA is repurified and heated after extract treatment, so that proteins will be removed and weak DNA bonding associations will be broken before analysis. However, we have found that the efficiency of the rejoining process varies with the break site. The 5-fold difference established for the rejoin efficiency of *Sal*I breaks and *Eco*RI breaks was supported by data for other break-sites. In part these data suggest that the cohesiveness of ends is important for rejoining efficiency, as seen for example by the low rejoining efficiency of *Hinc*II- (non-cohesive) or of *Acc*I-breaks (2-base overhang). It is possible that the *Eco*RI/*Sal*I difference is explained similarly, since *Eco*RI termini (5′ AATT overhang; 8 hydrogen bonds) will be more weakly associated than *Sal*I termini (5′ TCGA overhang; 10 bonds). However, the data for other break sites indicate that other factors, such as the polarity of the termini or possibly specific base-sequence and

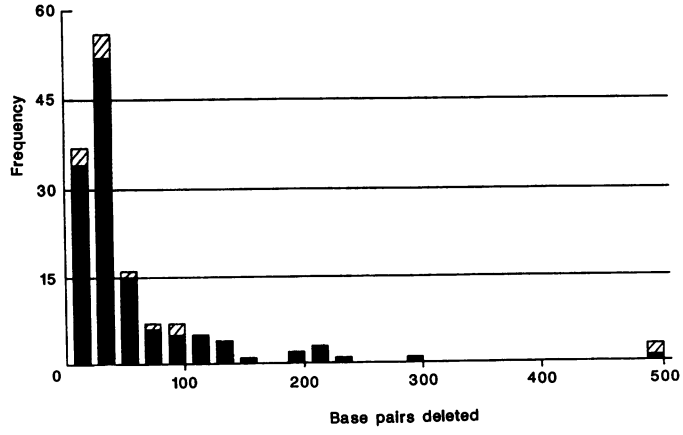

Fig. 5. Distribution of deletion sizes in mis-rejoined plasmids after extract-treatment and selection for lack of expression of the *lacZ* gene (white colonies). Closed bars, AT5BIVA extract-treated; hatched bars, MRC5V1 extract-treated.

topological location of these sites, may influence their response. Thus *Sal*I, *Bam*HI- and *Hin*dIII-breaks with 5′ 4-base overhangs were rejoined somewhat more efficiently than the 3′ -base overhangs given by *Pst*I- and *Sst*I-breaks. These data for the mammalian nuclear extracts contrast with the profile found for T4 ligase where, under the same reaction conditions, the rejoin efficiencies were: *Hin*dIII ≥ *Sst*I = *Pst*I > *Eco*RI > *Bam*HI > *Sal*I > *Acc*I (data not shown).

We have found, in agreement with our previous cellular studies (5), that extracts from transformed A-T and normal cells do not differ in rejoin efficiency. These results are in keeping with measurements showing similar DNA ligase activities in cells of these genotypes (28). We have now shown, in addition, that extracts from early-passage (diploid) human cells give similar relative efficiencies of rejoin at the different sites tested (Fig.4).

Rejoin fidelity

While nuclear extracts from the different cell types have similar rejoin *efficiencies*, we have found differences in the *fidelity* with which these extracts rejoin broken DNA at certain sites. Although it is known that bacteria will mis-rejoin a proportion of broken plasmid molecules (29; see also Table 2), high mis-rejoin frequencies were only found after human nuclear extract treatment. Extracts from the nuclei of the AT5BIVA cell line, in particular, give a highly significant increase in the loss of *lacZ* gene function when rejoining certain break-sites. It is notable that those break-sites (especially *Eco*RI and *Acc*I) which yield a low fidelity of rejoining with the A-T extract also have relatively low rejoin efficiencies. This suggests that where breaks are more difficult to rejoin, some extracts will act on the broken DNA to remove sequence.

However, the two 3′ 4-base overhang sites show similar 'intermediate' rejoin efficiencies but give quite different mis-rejoined fractions (Table 5), in one case (*Sst*I) similar to that for the site having the highest rejoin efficiency (*Sal*I). It is notable that these two endonucleases generate the same sequence at the overhang (5′ TCGA or 3′ AGCT), while the reverse of this sequence (*Hin*dIII break site, 5′ AGCT) does not show high fidelity rejoining. This result suggests that the nuclear extract may contain some very specific means of ensuring that certain sequences are rejoined faithfully.

It is known that broken DNA ends act as sites of action or recognition for enzymes which degrade or delete sequence. Simple mechanisms for lack of fidelity, such as an increased nuclease activity in the A-T extract, were not revealed by an increase in the extent of degradation of DNA on gels after treatment with A-T extracts, and no differences between cell lines were found in simple nuclease assays (data not shown). Alternatively, there may be lack of 'protection' of DNA ends in the A-T extract (5), allowing degradative processes access to the DNA. Such protection would arise minimally from the weak hydrogen-bonded association of the broken ends, more strongly from proteins which bind to the ends, and maximally from rapid ligation. DNA binding proteins protecting broken ends have been identified, for example, in infectivity studies of bacteriophage Mu (30) and in HeLa cells infected with adenovirus (31). Additionally, in studies of break rejoining in other organisms, such as *Escherichia coli* (32) and extracts from *Xenopus laevis* eggs (33), it has been suggested that 'accessory' (DNA binding) proteins are involved in the rejoining process. A defect in a complex that holds free ends together after endonucleolytic breakage has also been proposed in studies of aberrant immune gene rearrangements in mice suffering severe combined immune deficiency (34). However, we cannot at present comment on the relevance of our data to the genetic defect in the A-T disorder; extracts from only one A-T cell line were used and it will be necessary to test extracts from the cells of other patients before generalizations can be made.

ACKNOWLEDGEMENTS

This study was supported in part by the Commission of the European Communities contract B16-E-144-UK.

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