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Multiple components are involved in the efficient joining of double stranded DNA breaks in human cell extracts

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ABSTRACT

We describe a rapid and efficient *in vitro* system for the rejoining of double stranded breaks in DNA based on extracts of human 293 cells. Using this system as an assay, we have separated the nuclear extract into several components involved in break rejoining. The unfractionated system can convert approx. 100% of the input DNA, linearized with a restriction enzyme, to high molecular weight material at low temperature (17°C), and at the physiological temperature of 37°C we have shown that competing activities in the extract can also act on the DNA template. We present the fractionation of the extract and the partial purification of a novel factor which will stimulate a crude rejoin activity and in addition increases the activity of purified DNA ligase I. We have also partially purified the break joining activity and show that the chromatographic properties do not directly correspond with the three DNA ligases previously described, indicating that the activity observed may not be due to a single enzyme species. By studying the rejoining of double stranded DNA breaks as a biochemical process, we have demonstrated that the efficient joining of such breaks requires factors in addition to DNA ligases.

INTRODUCTION

Numerous studies have implicated the DNA double stranded break (dsb) as the critical lesion leading to a wide range of cellular endpoints such as chromosomal aberrations and cell death. In addition, such studies indicate that the majority of breaks introduced by agents such as ionizing radiation or treatment with restriction endonucleases, can be efficiently repaired by cells *in vivo* (for a review see Ref 1). In bacteria there is evidence that ligase mutants are extremely sensitive to dsbs induced by endonucleases (2), but mutants have been described both for prokaryotes and eukaryotes which although they show a defect in the repair of dsbs, do not appear to be deficient in DNA ligases (3,4,5). This implies that the process of joining dsbs may require factors in addition to these enzymes.

The ligation of single stranded nicks in synthetic double stranded DNA templates, has been well characterised and DNA ligases have been described from a variety of organisms (for a review see Ref 6). In bacteria and yeast, only one type of ligase has been identified as yet, although in higher eukaryotes multiple species have been found. Enzymes equivalent to the mammalian ligases I and II have been identified in *Drosophila melanogaster,* and recently a third DNA ligase, with properties distinct from the two described above, has been identified in humans (7).

The use of *in vitro* systems has been extremely important for the elucidation of many complex cellular processes in both procaryotes and eucaryotes. In the fields of replication (for a review see Ref 8), recombination (9) and excision repair (for a review see Ref 10) major advances have been made in recent years in the characterisation of these processes. Furthermore, the fractionation of crude extracts in combination with specific assay systems, has led to the identification of proteins with defined roles in each process.

A cell-free system for the repair of dsbs in human nuclear extracts has been previously described (11). Briefly, a plasmid molecule was linearized by treatment with a restriction endonuclease, incubated with a nuclear extract at 14°C, and the rejoined products detected either by bacterial transformation or Southern analysis. In this report, we describe a highly efficient system in which we have examined the rejoining of double strand breaks at low temperatures, and also at the physiological temperature of 37°C, in an attempt to more faithfully reproduce the *in vivo* process(es). In this system, which uses highly active extracts derived from human 293 cells, the rejoin reaction will convert approx. 100% of the input molecules to high molecular weight material at 17°C, while at 37°C a proportion of the DNA ends are modified by factors in the extracts making them refractory to ligation. We report the initial separation of the crude extract into three fractions one of which contains the majority of break joining activity and another which stimulates ligation. The fractionation profile of the break joining activity does not exactly correspond to the published protocols for ligase I, II or III suggesting that the observed break joining activity may contain mutiple components. The stimulatory factor (*r*ejoin *e*nhancing *p*rotein, REP1) has been purified over 500 fold and has been shown not only to stimulate the break join activity by 4–5 fold but also to increase the activity of purified ligase I when assayed in this system. This demonstrates for the first time *in vitro* that factors in addition to DNA ligases are involved in the process of joining dsbs in human extracts.

MATERIALS AND METHODS

Cells

Human 293 cells were propagated as suspension cultures in Ca^{2+}free MEM (Imperial) containing 10% newborn calf serum. 293 cells are a human embryo kidney cell line transformed with fragments of adenovirus type 5 DNA (12). The cell line was

adapted from monolayer culture (13) and was a gift from B.Stillman, Cold Spring Harbor, New York.

Plasmid preparation

The plasmids pUC18 and its derivative pSV011, which contains the *Hind* III-*Sph* I fragment of SV40 virus (14), were grown in *E.coli* HB101, isolated using alkali lysis and purified by double banding on caesium chloride-ethidium bromide gradients. Plasmids were linearized with restriction endonucleases as indicated and the digestion monitored by agarose gel electrophoresis. Only material which showed no forms other than linear molecules when 10 μg was analyzed on a gel, was used as a template in break-join reactions.

Preparation of cell extracts

The following procedure describes the isolation of nuclear and cytoplasmic extracts from a litre (approx. 5×10^5 cell per ml) of 293 cells grown in suspension. Cells were harvested by centrifugation and washed twice with cold phosphate buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g sodium phosphate [monobasic], 1.5 g sodium phosphate [dibasic] per litre). The cells were then washed in 5 ml cold hypotonic buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid [Hepes]-KOH [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol [DTT]) and finally resuspended in 2.5 ml of the same buffer. After 10 min on ice, the cells were disrupted by repeated Dounce homogenization until >90% of the cells were broken (20−30 strokes of a B pestle), and the cells left on ice for a further 30 min. The suspension was centrifuged (4,000 rpm in a Beckman JA-13 rotor for 10 min with half brake, 4°C) and the cytoplasmic extract removed. The nuclear pellet was resuspended by gentle vortexing in 20 ml cold buffer B (20 mM Tris HCl pH 7.5, 0.5 mM $MgCl_2$, 0.5 mM KCl, 0.5 mM DTT), containing 0.5% Triton X-100, recentrifuged, and the nuclei rewashed in 20 ml cold buffer B containing 1 mM phenylmethylsulphonyl fluoride (PMSF). The pellet was finally resuspended in 2 ml buffer C (500 mM NaCl, 2 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 10% sucrose) and the nuclei lysed by freeze-thaw cycles in liquid nitrogen. Debris was removed by centrifugation and the supernatant adjusted to 70% saturation with ammonium sulphate. The precipitate was allowed to form on ice for 30 min with stirring, and was recovered by centrifugation at 15,000 g for 20 min. The pellet was redissolved in 2 ml buffer D (25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM PMSF) containing 25 mM NaCl, and dialysed against 500 vols of the same buffer for approx. 18 h at 4°C. The cytoplasmic and nuclear extracts were aliquoted in small volumes, and snap frozen in a dry ice/ethanol bath. The extracts were stored at −70°C where they were stable for over 6 months. Aliquots taken for assay were not reused. Protein concentrations were approx. 8 mg/ml for the cytoplasmic extract and 4 mg/ml for the nuclear extract. Protein concentrations were determined using the Bio-rad protein assay reagent, against BSA as a standard.

Break-joining reactions

Standard reactions (25μl) contained (final concentrations) 70 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP pH 7.0, 1 μg linear plasmid DNA, and nuclear extracts or T4 DNA ligase (Pharmacia) as indicated. Reactions were prepared on ice and the reaction started by placing the tubes at the required temperature as indicated. Reactions were terminated by addition of an equal volume of 100 mM EDTA/1% SDS and the DNA purified by digestion with Proteinase K (2 mg/ml) and ribonuclease A (1mg/ml). The volume was adjusted to 200 μl with TE (10 mM Tris pH 7.5, 1 mM EDTA) and the samples extracted sequentially with phenol, phenol/chloroform (1:1), and chloroform followed by ethanol precipitation. The samples were resuspended in 20 μl of gel loading buffer and subjected to electrophoresis. Gels were 1% agarose, unless otherwise stated, in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA), and were run in the presence of 0.5 μg/ml ethidium bromide for 15−17 h at 2.25V/cm.

Quantitation of rejoined products

Gels to be quantified were photographed with Polaroid type 55 film and the negative processed with 18% sodium sulphite and air dried. Negatives were scanned using a Beckman DU-64 spectrophotometer and the results expressed as a percentage of DNA converted to rejoined forms.

Loss of terminal phosphate

10 μg of *Sal*I-cut pUC18 was dephosphorylated using calf alkaline phosphatase (Pharmacia), and 1 μg of this material 5′ end-labelled with γ^{32}P-ATP using T4 polynucleotide kinase (Pharmacia) using standard methods (15). Reactions were set up as described for break-joining but also contained 10,000 cpm of labelled plasmid. After incubation, the reactions were terminated by the addition of 10 mM EDTA containing 50 μg of denatured, sheared calf thymus DNA as carrier (final concentrations). 1 ml of 8% trichloroacetic acid (TCA)-1% pyrophosphate was added, and the samples left for 15 min on ice. The precipitate was collected by filtration onto Whatman GF-C filters and then washed twice with 10 ml cold TCA-pyrophosphate and once with cold ethanol. Filters were air dried and the incorporated radioactivity determined by liquid scintillation counting.

Redigestion of rejoined molecules

Rejoined products from reactions using a *Sal*I-cut template were treated as described above, but instead of resuspension in gel loading buffer the DNA was extracted using the GeneClean kit (BIO-101), ethanol precipitated, and digested overnight with 15 units/reaction of *Sal*I. Reactions were reprecipitated with ethanol, resuspended in gel loading buffer and subjected to electrophoresis as described above.

Fractionation of the 293 nuclear extract

The nuclear extract prepared from 3×10^9 cells (55 mg protein) was loaded onto a 15 ml phosphocellulose column equilibrated with buffer D containing 25 mM NaCl. The column was washed with 30 ml of the same buffer followed by subsequent washes of buffer D containing 0.4 M NaCl and finally with 30 ml buffer D containing 0.8 M NaCl. Protein was detected using the Bio-rad assay, the peaks were pooled and separately dialysed for 15 h against buffer D containing 25 mM NaCl plus 20% sucrose. Reactions containing 4.5 and 9 μg of each fraction alone, or 9 μg of each in combinations were assayed at 37°C for 15 min. To estimate the maximum effect of the stimulatory factor in fraction 3 on fraction 2 (see results) standard reactions were set up containing a subsaturating amount (2 μg) of fraction 2, with either 2,4, 6 or 8 μg of fraction 3. Reactions were incubated for 15 min at 37°C and the products analysed by gel electrophoresis. Gels were photographed and scanned as described to estimate the amount of rejoined products.

The 0.4 M phosphocellulose fraction (26 mg) was adjusted to 40% saturation with ammonium sulphate and stirred on ice for 30 min. The precipitate was collected by centrifugation (15,000 g for 20 min) and the supernatant adjusted to 70% saturation and treated as before. The resulting pellet was dissolved in a small volume of buffer D and dialysed against the same buffer containing 100 mM NaCl. This was then loaded on a native DNA cellulose column in buffer D containing 100 mM NaCl (12 mg) and the activity eluted with the same buffer containing 300 mM NaCl. The resulting protein (4 mg) was then loaded onto a second phosphocellulose column equilibrated in buffer D containing 0.2 M NaCl and the activity eluted at 800 mM NaCl The resulting breakjoining activity had a similar sensitivity to NaCl as the crude nuclear extract, was resistant to repeat freeze/thaw cycles and was stable at −70°C for at least 2 months.

Partial purification of the rejoining enhancing protein (REP 1)

The 0.8 M peak from phosphocellulose (12 mg) was loaded onto a 2.5 ml Q Sepharose column equilibrated with buffer D containing 0.2 M NaCl. The active fraction was eluted with the same buffer containing 1.0 M NaCl. This fraction was dialysed for 17 h against buffer D containing 25 mM NaCl and 20% sucrose. The resulting fraction was further purified by a 40−70% precipitation using a saturated solution of ammonium sulphate. The precipitate was dissolved in buffer D containing 25 mM NaCl and dialysed for 17 h against the same buffer. The partially purified material (0.2 mg, REP 1) was stable at −70°C for at least 2 months.

Effect of REP 1 on purified DNA ligase I

Samples of purified ligase I from calf thymus (a gift from T. Lindahl, ICRF Clare Hall Laboratories), REP 1, ligase plus REP 1 or ligase plus REP 1 which had been heated for 10 min at 90°C, were incubated at 37°C for 15 min and analysed as described. To estimate the amount of stimulation of REP 1 on ligase I, 100 ng of pure protein was incubated with 0.625, 1.25, 2.5 or 5 μg of REP 1 for 15 min at 37°C. The products were analysed by gel electrophoresis as described and the resulting gel photographed and scanned. The amount of activity caused by REP 1 alone was subtracted in each case.

RESULTS

Optimization of break-join reaction

It was preferable to use a cell line which grows as a suspension culture for the purification of factors involved in cellular processes because of the large number of cells required. The 293 cell line has been extensively used for fractionation of DNA replication proteins (see Ref. 16 and references therein), and has been found to support higher levels of SV40 replication *in vitro* than other transformed lines (13). Although the enzymes required for replication are nuclear, the original replication extract procedure was designed so that most proteins are leached into the cytoplasmic fraction. Therefore, nuclear and cytoplasmic fractions were prepared as described for replication studies, and tested for double strand break rejoin activity against *Sal*I-linearized plasmid at 17°C for 1 h. Over 90% of the rejoin activity was found in the nuclear fraction (data not shown), therefore this fraction was used for all further experiments. The addition of glycerol (10%) or polyethylene glycol 6000 (12.5%) to the reaction buffer did not increase the amount of rejoined products. The addition of NaCl to the extract resulted in a slight stimulation at 50 mM, while the activity was inhibited 50% at 150 mM and no rejoined products were observed at 200 mM. The reaction was also sensitive to $CaCl_2$ with a 50% inhibition of activity at 20 mM and no visible products at 100 mM. The break-join activity was destroyed by treatment with heat (90°C for 10 min) or treatment with proteases (2 mg/ml Proteinase K for 30 min at 37°C) thus indicating that a protein component is involved in formation of rejoined molecules. The reaction was completely dependent on the presence of a divalent cation with Mg^{2+} giving greater activity than Mn^{2+}. The omission of ATP in the reaction resulted in a 30−50% decrease in activity, but this residual activity is probably due to the presence of preformed AMP-ligase complexes in the extract (6). Pretreatment of the extract by dialysis against buffer D containing 1 mM sodium pyrophosphate, which would disrupt these complexes, followed by dialysis to remove the pyrophosphate, rendered the extracts essentially ATP dependent.

Comparison of break-join activity at 17 and 37°C

To examine the kinetics of the break-join process, reactions were set up with increasing amounts of nuclear extract at 17°C for 1 h. At low protein levels, linear dimers were observed followed by the presence of linear trimers and higher multimers (Fig 1A). At approx. 10−12 μg/reaction of extract, almost all of the template was converted into high molecular weight material which, under the gel conditions used, appears mainly as a single band of >12 kb. When this experiment was repeated at the physiological temperature of 37°C (Fig 1B), a similar pattern to that found at 17°C is seen, but the proportion of material in each band accumulates as extract levels are increased. The addition of higher concentrations of extract does not result in the conversion of these forms to higher molecular weight species. Addition of dNTP's to the reactions at concentrations of 10−50 μM had no affect on the forms produced. However, a titration of T4 ligase at 37°C did not show this distribution of forms, with the majority of template rejoined as high molecular weight material (Fig 1B lanes 2−4) indicating that this pattern is produced by components in the nuclear extract rather than simple ligation.

To examine this further, a time course was carried out at a protein concentration (10 μg/reaction), giving approx. 100% conversion of the template into rejoined products at 17°C. The appearance of a dimer band is seen after 1 min incubation at 17°C (Fig 2A lane 2), and by 60 min the majority of the products are high molecular weight species. At 37°C, although the reaction is initially faster than at the lower temperature (compare lane 4 in Figs 2A and 2B), the forms are again distributed between linear dimer, trimer and higher forms which, even after a 2 h incubation, are not converted to high molecular weight material. Quantitation of the gel in Fig 2B by densitometric scanning showed that, after a 2 h incubation at 37°C, over 80% of the input DNA is converted to rejoined products.

Analysis of rejoined products

To examine the high molecular weight rejoined products formed at the two temperatures, standard reactions were performed at 17 or 37°C and the products separated on a 0.66% agarose gel. As seen in Fig 3, the overall pattern of bands seen at the two temperatures is similar, with the high molecular weight band observed on 1% gels resolved into a series of forms, ranging from linear dimer (approx. 6 kb) to multimers of at least 10 monomer lengths (>30 kb). However the proportion of the forms

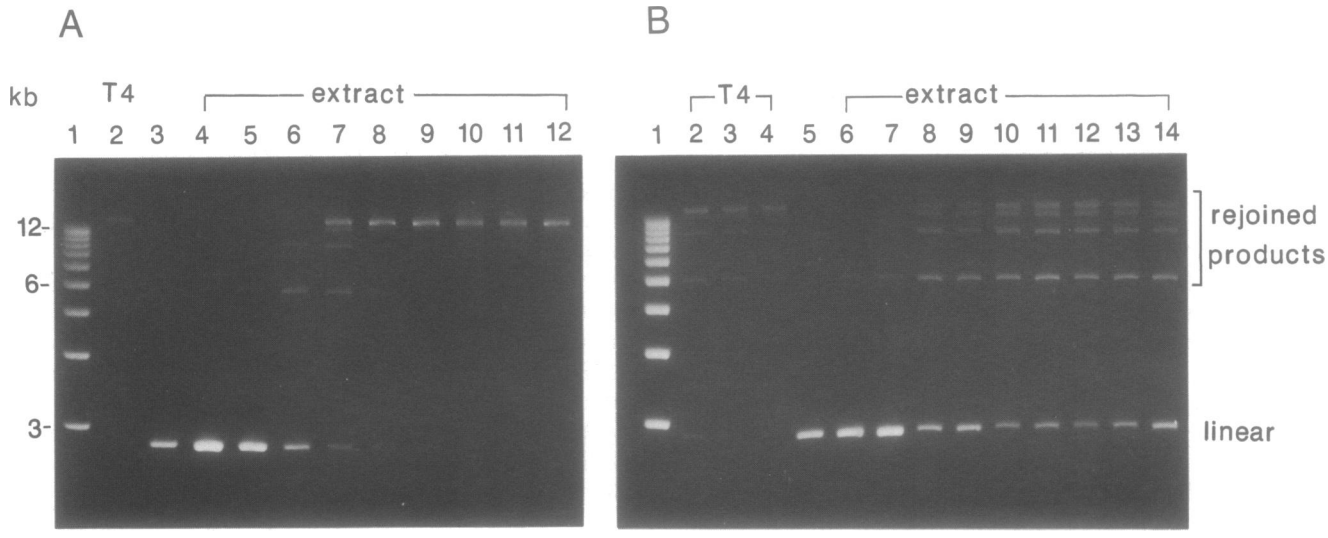

Figure 1. Effect of extract concentration on rejoined products. Reactions contained 1 μg *Sal*I-cut plasmid and increasing amounts of 293 nuclear extract in a final volume of 25 μl. (A) reactions incubated at 17°C for 60 min, (B) reactions incubated at 37°C for 15 min. (A) Lane 1, 1 kb ladder (BRL); lane 2, 1 unit T4 ligase; lane 3, no extract; lanes 4–12; 0.625 (2.5%), 1.25 (20%), 2.5 (38%), 5 (97%), 7.5 (98%), 10 (98%), 12.5 (98%), 15 (98%) and 20 μg (98%) of nuclear extract respectively. (B) Lane 1, 1 kb ladder; lanes 2–4 1, 2 and 4 units of T4 ligase; lane 5 no extract; lanes 6–14, 0.625 (1%), 1.25 (10%), 2.5 (32%), 5 (34%), 7.5 (76%), 10 (67%), 12.5 (71%) 15 (66%) and 20 μg (50%) of nuclear extract respectively. Figures in brackets indicate% template converted to rejoined products.

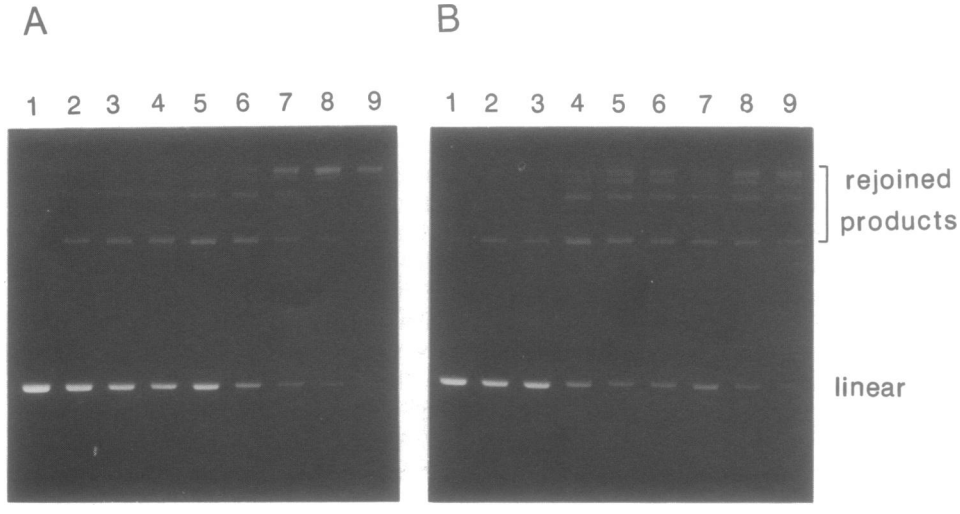

Figure 2. Time course of break joining reaction at 17 and 37°C. Reactions contained 1 μg of *Sal*I-cut plasmid and 10 μg of nuclear extract. Reactions were incubated at 17°C (A), or at 37°C (B). Lanes 1–9; 0,1, 2, 5, 10, 15, 30, 60, 120 min respectively. (A) lanes 1–9 show 0, 17, 18, 26, 33, 76, 72, 95 and 98% template converted to rejoined products respectively, (B) lanes 1–9 show 0, 20, 19, 31, 64, 74, 64, 78 and 88% template converted.

produced at the two temperatures differ, with reactions at 37°C showing that the products consist mainly of linear dimer and trimer bands, with little material over 12 kb.

The pattern observed at 37°C may be caused by a factor in the extract competing for the free DNA ends, thus removing them from the break joining reaction. As this phenomenon did not seem to be occurring at 17°C we attempted to 'chase' the pattern of products produced at 37°C to the high molecular weight form by a two stage reaction. Reactions containing 20 μg of extract were set up and incubated at 37°C for 15 min and the rejoined products purified as described in Materials and Methods. To this, either a further aliquot of extract or 1 unit of T4 ligase was added, and the reactions incubated at 17°C for 2 h. The DNA was re-isolated and the products examined by gel electrophoresis. The pattern of bands produced however, was identical to reactions which had been subjected to the first incubation alone (ie at 37°C, data not shown). This indicates that the DNA ends were not blocked by a protein component, which would have been removed by the deproteinization procedure, but that after a 15 min incubation at 37°C the DNA ends of a proportion of the input molecules were becoming unligatable.

To test this further, pUC18 linearized with *Sal*I was 5'end-labelled with γ^{32}P-ATP and added to standard rejoin reactions containing 1 μg of unlabelled *Sal*I-cut plasmid at 17 and 37°C.

Figure 3. Analysis of rejoin products by low% gel electrophoresis. Standard reactions containing 1 μg of *Sal*I-cut plasmid were incubated with 20 μg nuclear extract at 17°C for 120 min or at 37°C for 15 min and analysed by gel electrophoresis on a 0.66% agarose gel. Lane 1, 1 kb ladder; lane 2, reaction at 37°C; lane 3, reaction at 17°C.

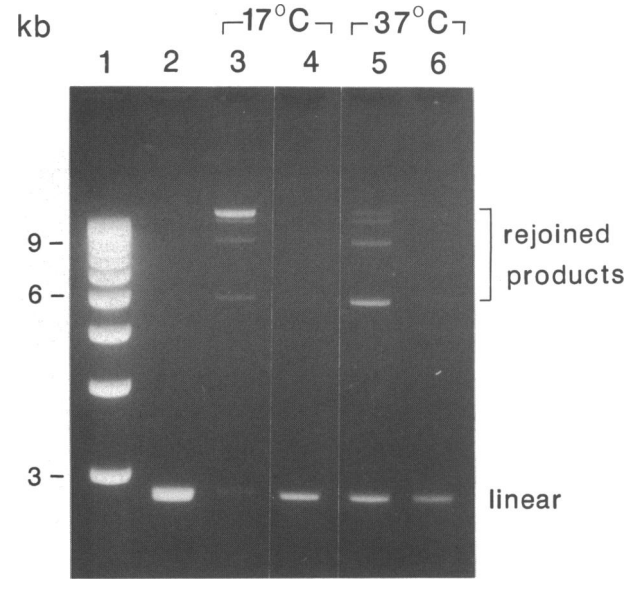

Figure 4. Redigestion of DNA products formed after break join reactions at 17 or 37°C. Standard reactions, in duplicate, containing 1 μg of *Sal*I-cut plasmid were incubated with 20 μg of nuclear extract at 17°C for 120 min or at 37°C for 15 min, and the DNA isolated as described in Materials and Methods. One sample at each temperature was then redigested at 37°C with an excess of *Sal*I endonuclease, and subjected to gel electrophoresis. Lane 1, 1 kb ladder; lane 2, 1 μg *Sal*I-cut plasmid; lane 3, rejoin products at 17°C prior to redigestion; lane 4, rejoin products at 17°C after redigestion with *Sal*I; lane 5, rejoin products at 37°C prior to redigestion; lane 6, rejoined products at 37°C after redigestion with *Sal*I. Note: the extended DNA purification procedure required for subsequent redigestion resulted in some loss of input DNA not seen in other gels.

Table 1. Removal of the terminal ^{32}P-residue by the nuclear extract at 17 and 37°C.

Treatment	% label remaining*
no incubation	100
− extract 17°C	87
+ extract 17°C	76
− extract 37°C	89
+ extract 37°C	42

* Average of two experiments. Error +/− 2%.

Reactions were set up with and without 20 μg extract and incubated at the two temperatures for 60 min and 15 min respectively and the amount of label remaining in association with the template measured. As can be seen in Table 1, at 17°C the presence of extract has a minimal effect on the amount of label removed (13%), while at 37°C the presence of extract in the reaction results in over half (58%) of the terminal phosphate being lost from the DNA template. This suggests that at 37°C a phosphatase or nuclease is active in the extract which is affecting the ends of the DNA template thus removing them from the rejoin reaction.

Estimation of mis-rejoining at 17 and 37°C

Previous work has indicated that nuclear extracts have low levels of mis-rejoin of *Sal*I-linearized plasmid after incubation, as estimated by a transformation assay (11). We therefore wanted to test whether the amount of mis-rejoining of monomer units was significantly altered when using the more efficient system described here, and also whether the increase in temperature resulted in a reduction in fidelity of rejoining. If the multimers produced were formed without base changes, then recutting with the enzyme which was used to generate the linear template originally, should result in the reappearance of monomer sized material. As seen in Fig 4, the DNA products produced at both 17 and 37°C were redigestable to monomer size lengths. This indicates that the majority of the DNA products, formed at either temperature, are rejoined faithfully by the system described here and therefore this could be used as a suitable assay for the normal rejoin process. A transformation assay as described previously (11) using the 293 cell extract also showed low (<0.2%) levels of mis-rejoined molecules (data not shown).

Fractionation of the 293 nuclear extract

To try to identify components that are required for the rejoining of double strand breaks in this system, we have separated the 293 extract into several fractions. The nuclear extract was initially divided by phosphocellulose chromatography into three components as described in Materials and Methods. Fraction 1 (25 mM NaCl) contained no rejoin activity, fraction 2 (400 mM NaCl) showed the majority of rejoined forms (linear dimers, trimers and higher multimers) while fraction 3 (800 mM NaCl) contained less rejoin activity (dimer band only, Fig 5 lanes 9 & 10). Combinations of the three fractions however, showed that at equal protein concentrations, fraction 3 has a stimulatory affect on the activity present in fraction 2 (Fig 5 compare lane 7 [4.5 μg fraction 2 alone] and lane 9 [4.5 μg fraction 3 alone] with

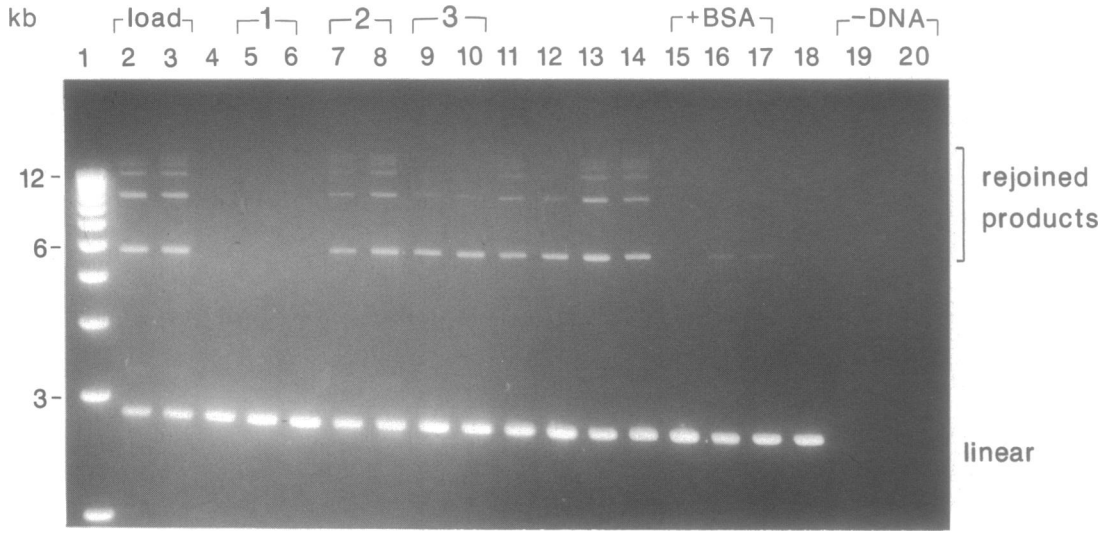

Figure 5. Fractionation of the 293 extract. The nuclear extract was divided by phosphocellulose chromatography as described, into three fractions. Samples (4.5 and 9 μg) of each peak at were incubated with 1 μg of *Sal*I-cut plasmid on their own (lanes 5–10), or at a level of 4.5 μg, in combinations of fractions (lanes 11–14). All reactions were incubated at 37°C for 15 min. Lane 1, 1 kb ladder; lanes 2 and 3, 4.5 and 9 μg of unfractionated nuclear extract; lane 4, no added extract; lanes 5 and 6, 4.5 and 9 μg of fraction 1 (25 mM NaCl); lanes 7 and 8, 4.5 and 9 μg of fraction 2 (400 mM NaCl); lanes 9 and 10, 4.5 and 9 μg of fraction 3 (800 mM NaCl); lane 11, 4.5 μg of fraction 1 plus 4.5 μg of fraction 2; lane 12, 4.5 μg fraction 1 plus 4.5 μg of fraction 3; lane 13, 4.5 μg of fraction 2 plus 4.5 μg of fraction 3; lane 14, 4.5 μg of all three fractions together; lanes 15–17, 4.5 μg of each fraction separately plus 4.5 μg BSA; lane 18, 4.5 μg of all three fractions heat treated at 90°C for 10 min before incubation; lane 19, 4.5 μg of each fraction incubated without addition of template DNA; lane 20, 4.5 μg of all three fractions heat treated at 90°C without addition of template DNA.

lane 13 [4.5 μg of each fractions 2 and 3]), giving an amount of rejoined products which is greater than that produced by double the quantity (9 μg) of fraction 2 (compare lanes 8 and 13). The addition of BSA to each fraction cannot substitute for the stimulatory effect and appears to be slightly inhibitory (lanes 15–17).

Effect of stimulatory factors on break rejoining

Using the crude phosphocellulose fractions 2 and 3, it was found that the level of rejoined molecules could be increased from 7% (fraction 2 alone) to a maximum of 31% when an optimum amount of fraction 3 was present (data not shown). To determine if this effect was caused by a direct stimulation on ligase I, further experiments were performed with the partially purified material derived from fraction 3 (designated REP 1, see materials and methods) on ligase I. As shown in figure 6A, the level of ligase I used (100 ng) does not show activity in this assay (lane 1) but on addition of REP 1 a significant amount of rejoining is observed (lane 2). Increasing the amount of REP 1 increases the level of rejoining (Fig 6B) while increasing the level of ligase I to 250 ng in the absence of REP 1 still showed no visible activity in this assay (data not shown). Replacing REP 1 with an equal amount of BSA or the addition of BSA to the assay had no effect, nor did addition of REP 1 which had been heat treated for 90°C for 10 min (lane 3).

DISCUSSION

In this report we have described a highly efficient system for the rejoining of DNA double-strand breaks in nuclear extracts derived from human 293 cells. This increase in sensitivity has allowed us to demonstrate that the system can be used as an assay for the identification of components that are required for the rejoining process by biochemical fractionation (see below). We initially optimized the break rejoining at 17°C, but as our interest is to examine the break-joining process under physiological conditions we also examined the process at the higher temperature of 37°C. At 17°C, essentially all of the DNA template is used in the break joining reaction, while at the higher temperature it became obvious that competing activities in the extract were modifying a proportion of the DNA ends. However the basic characteristics of the break join system at the two temperatures were similar; the amount of mis-rejoin was not significantly different and also the relative efficiency of end rejoining between DNA with different end structures was the same. For example, a greater amount of rejoining was observed with DNA ends with a 4 bp overhang than with a 2 bp overhang than with blunt ends at either temperature (data not shown). As we were attempting to identify interactions between protein factors, all assays of the fractionated material were preformed at 37°C.

We have separated the nuclear extract into two major components, one of which contains the break joining activity and another fraction which stimulates this process 4–5 fold. Partial purification of the rejoining fraction indicates that the properties of our observed activity do not exactly correspond to the published purification of DNA ligase I (7,17) II or III alone (7). For example, the break joining activity elutes between 0.2–0.4 M NaCl on phosphocellulose whereas the majority of ligase I activity elutes at 0.2 M NaCl on this resin (7), and ligase II is removed at 0.7 M NaCl (7). Although the newly described enzyme ligase III is removed at this salt concentration (0.2–0.4 M NaCl), the break joining activity is precipitated at a higher ammonium sulphate concentration to that used in ligase III purification and in addition does not share the extreme sensitivity to NaCl as pure ligase III (7). We believe therefore that the activity we observe, which has been separated from the stimulatory factor, may not

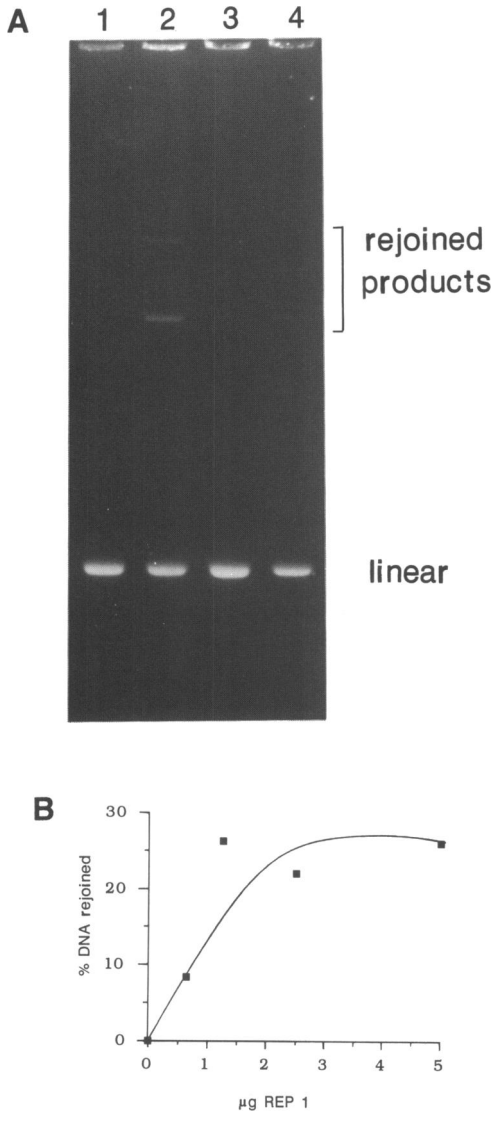

Figure 6. Effect of REP 1 on purified ligase I. A) Samples containing 1 µg of *Sal*I-cut plasmid were incubated with 100 ng of ligase I (lane 1), 100 ng ligase I + 2.5 µg of REP 1 (lane 2), 100 ng ligase I+2.5 µg of heat treated REP 1 (lane 3) or 2.5 µg of REP 1 alone (lane 4). Reactions were incubated at 37°C for 15 min. B) 1 µg of *Sal*I-cut plasmid was incubated with 100 ng of ligase I with 0, 0.625, 1.25, 2.5 or 5 µg of REP 1. Reactions were incubated at 37°C for 15 min and the products analysed by gel analysis. The gel was photographed and scanned to determine the percentage of rejoined products.

be simply attributed to any one of the described DNA ligases singly, but maybe that the activity is caused by one or more of the DNA ligases, perhaps with the assistance of other factors. We are currently in the process of further purification and characterization of this break joining activity to elucidate the factors involved.

We have also purified 500-fold a heat sensitive factor (REP 1) which stimulates the break joining process 4−5 fold. In an attempt to characterise the activity of this factor we replaced the break joining activity derived from the 293 extract with purified ligase I. In the presence of REP 1, the activity of this enzyme on a double stranded break is greatly enhanced (Fig 6 A and B). However as the pure ligase alone does not show activity under these conditions, we cannot calculate the fold stimulation caused by REP 1. Two stimulatory factors have been described which affect purified ligase I; one of these was from a normal human transformed line (18) which the authors later concluded was a non-specific effect caused by soluble protein (19). The second factor was identified from a transformed cell line derived from a patient with Bloom's syndrome and appears to be over-expressed specifically in this line. (20). This factor however differs from REP 1 in two respects; is resistant to heat (100°C for 10 min) while we show that REP 1 is sensitive to a similar treatment (Fig 6A) and REP 1 does not exhibit the same binding properties to single stranded DNA-cellulose chromatography as the factor described. Therefore, we do not think that REP 1 is the same as those stimulatory factors previously identified.

An *in vitro* system based on extracts derived from eggs of the amphibian *Xenopus laevis* has been described (21, 22) which will join ends of duplex DNA that differ in structure and sequence. Whole extract or a partially purified ligase (PPL) fraction will produce both open and closed circular molecules on cohesive ended templates but the PPL fraction will not produce open or closed circular forms on molecules which contain one blunt and one 3′ protruding single strand. This has led to the suggestion that an 'alignment protein' is involved in bringing the DNA ends together before infill and subsequent ligation (22). Experiments to determine whether REP 1 may have the properties of an alignment protein are in progress. It is interesting to note that we observe very little open or closed circular forms at either temperature when using cellular extracts. This is in direct contrast to samples joined by T4 DNA ligase, which under identical conditions, routinely produce circular forms (Fig 1B lanes 2−4, band between 3 and 4 kb markers). This suggests that the complete extracts may contain a factor which promotes the formation of linear multimers relative to circular forms.

The use of templates that are tailored to the properties of specific enzymes (eg the use of nicked DNA to monitor polymerase α activity, and synthetic oligomers for the detection of the DNA ligases) has been extremely useful for the characterization and isolation of such enzymes. However, it is becoming apparent that many fundamental processes are mediated by multi-enzyme complexes, which can only be detected using functional assays that reflect the overall process. For example, the identification of the single stranded DNA binding protein, RP-A which is essential for DNA replication, was identified by the fractionation of cellular extracts and by the use of such a functional assay (23). By the use of the break joining system described, we hope to identify factors other than DNA ligases that are involved in the rejoin process.

In summary, by studying the process of break joining in a simple *in vitro* system, we have identified components which together with DNA ligases result in the efficient rejoining of double strand breaks. The purification of such factors and the characterization of their properties, will be essential for the elucidation of the process of double strand break repair *in vitro* and *in vivo*.

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