Mismatch and blunt to protruding-end joining by DNA ligases

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ABSTRACT

A nuclear DNA ligase activity from immature chicken erythrocytes, and to a lesser extent T4-induced DNA ligase, can join cohesive-ends (3 and 5-nucleotides long) having one of the mismatches, A/A, T/T, C/C, G/G, at the middle position. The rate of ligation depends on the length and stability of the mispaired intermediate (G/G, T/T > A/A, C/C). When the non-complementary overhanging-ends are short (i.e. 1-nucleotide) both ligases catalyze the joining of the single-stranded protruding-end with a blunt-end. This reaction occurs at low but significant rates compared to blunt-end ligation. The chicken ligase has lower flush-end joining activity than T4 DNA ligase, but it is more permissive since it joins C/C or A/A mismatched-ends, whereas the prokaryotic ligase does not. Possible biological implications of the reactions are discussed.

We have also found that BstEII easily cleaves at sites harboring a C/C or a G/G mismatch at the center of its recognition sequence, whereas AvaiI (T/T or A/A), HinfI (G/G) and Ddel (G/G) do not.

INTRODUCTION

DNA ligases catalyze phosphodiester bond formation between adjacent 5'-phosphoryl and 3'-hydroxyl groups in DNA. This reaction is essential in such basic processes as DNA replication, repair and recombination. Consequently, DNA ligases, with rather similar properties, have been identified in prokaryotic and eukaryotic cells (for reviews, see refs. 1-3).

Although there has been some controversy as to whether mammalian DNA ligases exist as two distinct forms in vivo, it appears that, as opposed to yeast (4), higher eukaryotes contain two independent types (I and II) of the enzyme (5-7). It has recently been reported that DNA ligase I (but not II) can catalyze blunt-end joining, whereas DNA ligase II (but not I) can join fragments of oligo(dT) hydrogen-bonded to poly(rA) (8).

Given the plasticity of the genome, we were interested in exploring the ability of ligases to join DNA molecules independently of the type of ends present as substrates. Previous observations suggest that, under certain conditions, T4-induced DNA ligase can be forced to catalyze the joining of
hydrogen-bonded duplexes containing mismatched nucleotides (9, 10), as well as gapped circular DNA molecules (11). In this work we wanted to determine whether T4 DNA ligase can join mismatched protruding-ends of different length and whether eukaryotic ligases can, given the opportunity, perform similar types of reactions. This question is relevant both to the understanding of the mechanism of enzyme action and from the genetic viewpoint. If ligases were promiscuous, mismatches could arise during genetic recombination. The rate of fixation of the ensuing mutations could be as high as 50% since, regardless of the type of mismatch, the repair mechanisms of the cell may not know which strand to repair. Therefore, it is pertinent to determine if DNA ligases can incorporate non-complementary bases into duplex DNA.

We demonstrate that a nuclear DNA ligase activity from chicken, and to a lesser extent T4-induced DNA ligase, can efficiently generate a variety of mispaired molecules. We also show that BstEII is able to cleave sites containing mismatches in the center of its recognition sequence.

EXPERIMENTAL PROCEDURES

MATERIALS

Restriction nucleases were purchased from Boehringer Mannheim (AvaII, BstEII, HinFI, NaeI, NdeI), BRL Inc. (AccI, DdeI, PvuII, SmaI, TaqI), and New England Biolabs (XmnI, BatNI). T4 DNA ligase was purchased from BRL Inc. Plasmid pGEM3 was purchased from Promega Biotec.

DNA

Fragments were obtained by digestion of different plasmid DNA (pBR322, pGEM 3, p554 (13), pCHV2.98/H (14)) with the restriction nuclease studied. Appropriate fragments were purified by electrophoresis in low melting point agarose gels (BRL Inc.). The isolated DNA was treated with calf intestine alkaline phosphatase (Boehringer, Mannheim) and labeled at the 5' ends with T4 polynucleotide kinase (BRL, Inc.) and (γ32P)ATP (5-7x10^3 Ci/mmol, ICN, Irvine) as described (15).

Ligase preparations

A 0.3 M KCl nuclear extract from purified immature chicken erythrocytes or calf thymus, was prepared as previously described (16). Ammonium sulfate was added to the extract to achieve 30% saturation. After standing 15 min on ice, the precipitate was removed by centrifugation and ammonium sulfate to 60% saturation was added to the supernatant. The precipitate was dissolved in buffer A (10% glycerol, 5 mM MgCl2, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.2 mM EGTA, 20 mM Hepes, pH 7.9) containing 0.05 M KCl, dialyzed extensively.
against the same buffer, cleared by high speed centrifugation, and stored at -80 °C. The protein concentration was approximately 5 mg/ml.

The ligase activity was partially purified by column chromatography. Crude ligase was loaded onto a cellulose phosphate (Pll, Whatman) column equilibrated with 0.05 M KCl in buffer A, and eluted with a salt gradient (0.05 M -0.5 M KCl). Fractions with ligase activity were pooled, dialyzed against 0.1 M KCl in buffer A, and loaded onto a hydroxylapatite column. The column was washed with 0.3 M KCl in buffer A and the ligase activity was eluted with the same buffer containing 0.075 M sodium phosphate (pH 7.8). The partially purified ligase was dialyzed against 0.05 M KCl and 40% glycerol in buffer A, and stored at -20 °C.

**Ligase reactions**

The standard reactions (20 μl) were carried out for 1 h at 25 °C in buffer B (5 mM MgCl₂, 20 mM KCl, 0.2 mM EGTA, 5 mM dithiothreitol, 10 mM Hepes, pH 7.9) and contained 100-200 ng of DNA fragments (3-5x10⁴ cpm). The reactions with T4 DNA ligase (1 unit) contained 0.1 mM ATP, and those with chicken ligase (2 μl) contained 2 mM ATP. These concentrations of ATP were found optimal under our conditions. The reaction was quenched with EDTA (to 10 mM) and SDS (to 0.2%). Proteinase K (Boehringer, Mannheim; 10 g/reaction) was then added and the incubation was continued for 1 h at 50 °C. DNA was further deproteinized with phenol/chloroform and concentrated by ethanol precipitation.

**Analytical methods**

The ligation products were analyzed after secondary cleavage with restriction nucleases and/or alkaline phosphatase treatment as shown in Fig. 1. In a typical experiment, the products of ligation were digested for 2 h with 2 units/μg DNA of the appropriate restriction nuclease in a final volume of 20 μl. 1-2 μg of pBR322 or pGEM3 were always included in the reaction as internal controls of the digestion. DNA was fractionated by electrophoresis in 0.8% agarose gels in 0.5X TBE (50 mM Tris, 41 mM boric acid, 0.5 mM EDTA). For two-dimensional electrophoresis, 3 mm-wide strips were cut from tracks of the first dimension gel run in 0.5X TBE. The strips were soaked in excess 30 mM NaOH, 2 mM EDTA for 1 h and glued to the top of a 0.9% agarose gel made in 30 mM NaCl, 2 mM EDTA. The electrode buffer for the second dimension (30 mM NaOH, 2 mM EDTA) was re-circulated during electrophoresis.

Before autoradiography, the gels were treated for 1h with ice-cold 5% trichloroacetic acid and dried. Kodak XAR-5 films were exposed at -80 °C.

DNA concentrations were determined by fluorimetry (17).
RESULTS

Nuclear chicken ligase

The immature chicken erythrocyte contains a ligase activity that can be extracted from nuclear ghosts by 0.3 M KCl. The activity was assayed in crude extracts and after partial purification (300-400 fold, see Experimental Procedures). Similar results were obtained with both preparations. For simplicity, the term ligase is used in the singular without prejudice about the number of different types of ligases that might be present (see Discussion). The ligase is inactivated by a 10 min incubation at 50 °C or by 0.5 % SDS. Although ATP stimulates the reaction, the partially purified enzyme shows substantial activity without added ATP. This suggests that the ligase exists in an activated form (i.e. adenylate intermediate, 18). The activity of commercial T4-induced DNA ligase, on the other hand, is dependent on exogenously added ATP.

Strategy to characterize the ligation of mismatched DNA ends, and the ability of restriction nucleases to cleave at mispaired recognition sequences.

The general strategy is summarized in Fig. 1. It is based on the use of DNA fragments (I, a and a') having at one terminus a blunt-end (R2, Table 1) and at the other a 5' overhanging-end created by restriction nucleases of 'relaxed' specificity (R1). The R1 nucleases used (BatEII, AvaII, DdeI, MinFI, BatNI) recognize degenerate sequences containing an odd number of base pairs (bp). The DNA fragments are labeled at the protruding 5'-phosphate (R1 end).

A priori, dimers (II) obtained by separate ligation of individual fragments (a or a') can originate from cohesive-end (b) or blunt-end (c) joining. Because the 5'-overhangs have an odd number of base pairs, the products of cohesive-end ligation will have a mismatch at the center of the original R1 recognition sequence. The mispaired sites may or may not be cleaved by R1 (incertitude indicated by (?) in Fig. 1). If R1 activity is not affected by the mismatch, the two types of dimers can be distinguished by digestion with R1, R2 or R1 + R2 (III and IV). However, if the mismatched sequence is refractory to R1 cleavage, the nature of the dimers can be ascertained by treatment with alkaline phosphatase (V). This will remove the label from only those molecules joined through their blunt-ends. Alternatively, the products of the ligation can be digested with R3 (VII), a restriction nuclease that generates labeled fragments, the length of which (d or e) is diagnostic of the type of the ligation event. Further, to determine whether the resistance to cleavage is due to the mismatch or to the presence of nicks at the site of ligation, the quality of the reaction products can be
Fig. 1.- Strategy to study the nature of the products of ligation. (•) Radioactively labeled 5' protruding ends. R1, R2, and R3 indicate the cleavage sites of the different restriction nucleases used. R1 produces cohesive-ends and R2 flush-ends. ($) Ligated molecules harboring a mismatch; (—c) blunt-end ligated fragments. See the text for more details.

Finally, we have ensured that the concentrations of the restriction nucleases used are sufficient for complete digestion. This was determined by monitoring the digestion of non-labeled DNA mixed with the products of ligation.

Table 1 summarizes the different ends and mismatches examined, as well as the restriction nucleases used in the analyses.

**Ligases can join complementary 5-nucleotide long ends (BstEII) containing a (G/G) or a (C/C) mismatch at the center.** BstEII cleaves the resulting mispaired sequences.

When the chicken DNA ligase activity is presented with fragments containing blunt ends (PvuII) and either a GTGAC- or a GTCAC- protruding-end
TABLE 1. LIGATION OF MISPAIRED COHESIVE ENDS. SUMMARY OF THE MISMATCHES TESTED AND RESTRICTION NUCLEASES USED.

<table>
<thead>
<tr>
<th>R1</th>
<th>RECOGNITION SEQUENCE</th>
<th>R2</th>
<th>R3</th>
<th>MISMATCHES TESTED</th>
<th>LIGATION</th>
<th>CLEAVAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvaII</td>
<td>G G( )CC</td>
<td>Real</td>
<td>AccI, TaqI</td>
<td>A/A, T/T</td>
<td>yes(^a)</td>
<td>no</td>
</tr>
<tr>
<td>DdeI</td>
<td>C T(N)AG</td>
<td>NaeI</td>
<td>-</td>
<td>G/G</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>HinfI</td>
<td>G A(N)TC</td>
<td>SmaI</td>
<td>-</td>
<td>G/G</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>BatNI</td>
<td>CC↓ A( )GG</td>
<td>Real</td>
<td>AvaII, XmnI</td>
<td>T/T, A/A</td>
<td>no(^b)</td>
<td>-</td>
</tr>
<tr>
<td>BatEII</td>
<td>GT( )AC</td>
<td>PvuII</td>
<td>-</td>
<td>G/G, C/C</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

\(^a\) A/A mismatched ends not joined by T4-induced DNA ligase.
\(^b\) Ligation between BatNI - Real ends observed.

produced by BatEII, a significant number of dimers are generated (Fig. 2A and 2B, lanes 1). Subsequent digestion with BatEII, PvuII, or a combination of both, indicates that the main ligation event took place, almost exclusively, through the BatEII ends with little blunt-end joining (cf. Fig. 2A and 2B, lanes 2, 3, and 4; the electrophoretic mobility of the dimers in lanes 3 is distorted by the presence of indicator DNA fragments in that region of the gel). Two-dimensional gel electrophoresis showed that both DNA strands were sealed after joining (not shown, see Fig. 4E). Similar results were obtained with a partially purified calf thymus DNA ligase (not shown). Hence, the ligase can efficiently accept the mispaired ends, and BatEII can cleave the duplex molecules containing the mismatch G/G (Fig. 2A) or C/C (Fig. 2B) at the center of its recognition sequence. Thus, neither enzyme appears to be much affected by the distortion of the DNA helix.

T4 DNA ligase is also able to perform similar reactions (Fig. 2, D and E). However, the relative rate of the C/C mispaired ligation is much lower than in the case of the chicken ligase, and lower than blunt-end joining (cf. Fig. 2E, lanes 2 and 3). Addition of T4 DNA ligase to a chicken ligase reaction did not result in a higher production of the C/C mismatch (not shown), suggesting that the permissiveness of the eukaryotic ligase is not due to factors present in the enzyme preparation. Therefore, the phage-induced ligase appears to be more restrictive than its eukaryotic counterpart and, as
Fig. 2.- Ligation of BatEII-ends containing a G/G or a C/C mismatch at the center of the recognition sequence. Plasmid p554 was cut at the unique BatEII (R1) site and the linear molecules were labeled at the 5' ends. Secondary cleavage at a unique PvuII (R2) site generated two fragments of 1938-bp (a) and 3254-bp (a'). The 1938-bp fragment (B and E) carrying the protruding *GTCAC-end, the 3254-bp fragment (A and D) carrying the protruding *GTGAC-end, or an equimolar mixture of both (C and F) were reacted with chicken (A, B, and C) or T4-induced (D, E, and F) DNA ligase. The products of the reaction before or after cleavage with the indicated restriction nucleases were analyzed by electrophoresis in 0.8% agarose gels and detected by autoradiography. Lanes 0, mock reactions (i.e. no ligase added); lanes 1, products of the ligation; lanes 2, digestions with BatEII (R1, Fig. 1); lanes 3, digestions with PvuII (R2, Fig. 1); lanes 4, digestions with R1 + R2; lanes 5, mock double digestions (i.e. no enzymes added). Monomers (a and a') and dimers (b/c from a, or b'/c' from a') as in Fig. 1. The dot on the recognition sequence indicates the location of the mismatch.

It has been noted before (1, 19), it has a higher blunt-end joining activity (see also below).

The higher ability of the chicken ligase to join mismatched sequences was also tested in competition experiments, by comparing the relative yields of the products obtained by ligation of equimolar amounts of perfectly matching
Fig. 3.- Ligation of AvaII-ends containing a T/T or a A/A mismatch at the center of the recognition sequence. (A) A 1220-bp (a'), or (B) a 523-bp (a) AvaII-RsaI fragment from pBR322 labeled at the 5' protruding AvaII ends (GAC- and GTC-, respectively) were incubated with chicken ligase. The products of the ligation were analyzed as in Fig. 2. Lanes 1, mock reactions; lanes 2, products of the ligation; lanes 3, digestions with AvaII (R1); lanes 4, digestions with RsaI (R2); lanes 5, digestions with R1 + R2; lanes 6, mock double digestions. R1, R2, a, b, c as in Fig. 1.

BstEII ends. It is clear from Fig. 2C that the chicken ligase joins all possible combinations of protruding ends, albeit at different rates (i.e. G/C > G/G > C/C). In turn, T4 DNA ligase joined G/C and G/G ends, while those containing the C/C mismatch were not ligated to a detectable extent under the conditions of the reaction (Fig. 2F, cf. lanes 2 and 3). The relatively lower yields of mispaired dimers can be easily explained by the lower stability of the mispaired intermediates. The results strongly suggest that T4 DNA ligase is more affected by the structural deformation introduced by the mismatch than the chicken ligase.

We conclude that ligases can produce mispaired molecules harboring a G/G or a C/C mismatch two base pairs away from the site of ligation. Under the conditions of the reaction, the efficiency at which these events occur in vitro depends on the type of mismatch (G/G > C/C) and varies with the type of enzyme. Further, we conclude that BstEII readily cleaves molecules containing those types of mismatches at the center of its recognition sequence.
Fig. 4.- T4 DNA ligase is less permissive than chicken ligase and has a much higher blunt-end joining activity. (A and B) the 1220-bp (a') or (C, D, and E) the 523-bp (a) AvaII-RsaI fragments used in the experiments described in Fig. 3 were incubated with chicken (A and D) or T4-induced DNA ligase (B, C, and E). The products of the reaction were analyzed as in Fig. 2. Lanes 1, products of the ligation; lanes 2, digestions with TaqI (R3) (A and B) or AccI (R3) (C to E); lanes 3, digestions with AvaII (R1) + the respective R3; lanes 4, alkaline phosphatase treatment; lanes 5, alkaline phosphatase treatment and digestion with AvaII (R1) + RsaI (R2). (E) Analysis by two-dimensional gel electrophoresis of the DNA fragments from the experiment shown in C, (lane 3). CCC, covalently-closed circular molecules, R1 to R3 and a to e as in Fig. 1.

Ligases can join non-complementary 3-nucleotide long protruding-ends.

To determine to what extent the length of the complementary sequence influences the rate of mismatch-end joining, the ability of ligases to join overhangs of shorter length was examined. In these experiments, the fragments were generated by one of several R1 nucleases that produce 3-nucleotide long cohesive ends (Table 1).

The results obtained by ligation of fragments containing one AvaII end (G(A/T)C-) (R1) and one RsaI-end (R2), where the possible production of dimers carrying an A/A or T/T mismatch was investigated, are shown in Figs. 3 and 4. Clearly, the chicken ligase readily joined the monomers creating dimers and oligomers (the additional bands, shorter than monomer length in Fig. 3A are due to degradation by endonucleases contaminating the ligase preparation). Digestion with AvaII or RsaI (Fig. 3A and 3B, lanes 3-5) indicates that most dimers are refractory to both restriction nucleases. RsaI did cleave, however, the trimers (Fig. 3B, lanes 4 and 5). This is expected since these molecules must necessarily be the product of a blunt-end and a protruding-end ligation. The resistance of the dimers to cleavage, and the fact that the internal indicator DNA was completely digested with either restriction
Fig. 5.- Ligation of DdeI and HinfI-ends containing a G/G mismatch at the center of the recognition sequence. (A) A 790-bp DdeI-NaeI fragment from pCHV2.5B/H labeled at the 5' protruding DdeI end (\textsuperscript{5}'TGA-), or (B) a 920-bp HinfI-SmaI fragment from pCHV2.5B/H labeled at the 5' protruding HinfI end (\textsuperscript{5}'AGT-) were incubated with chicken ligase. The products of the ligation were analyzed as in Fig. 2. Lanes 1, mock reactions; lanes 2, products of ligation; lanes 3, digestions with (A) DdeI (R1), or (B) HinfI (R1); lanes 4, digestions with (A) Nae I (R2), or (B) SmaI (R2); lanes 5, digestions with R1 + R2; lane 6, mock double digestion. R1, R2, a, b, and c as in Fig. 1.

nuclease (not shown), suggested that these molecules are ligated via the protruding AvaII ends, thus, containing a A/A (Fig. 3A) or a T/T (Fig. 3B) mismatch. That this is the case, is shown in Fig. 4 (A and D). For these analyses, the respective products of ligation were digested with R3 (AceI or TaqI). Dimers containing a A/A or T/T mismatch should generate labeled fragments of 1856-bp and 974-bp in length, respectively. Fig. 4 (A and D, lanes 2 and 3) shows that the predicted fragments are, indeed, produced. Moreover, the radioactive 5' phosphate of the mismatched dimers should be resistant to alkaline phosphatase treatment, whereas it should be labile in those dimers joined via the blunt-ends. Figure 4D (lanes 4 and 5) demonstrates that this premise is also met.

A different result was obtained with T4 DNA ligase. TaqI (R3) digestion of the ligation products indicates that virtually no joining of the A/A mismatched ends occurred, most of the dimers originating from blunt-end
Fig. 6. - Overhanging-end to flush-end ligation. A 1213-bp (A, C, C', and E) or a 644-bp (B, D, and D') BstNI-RalI fragments of pGEM3 labeled at the 5' protruding BstNI-ends (*T-), and (*A-), respectively, were incubated with T4 DNA ligase (A, B, C, D, and E) or chicken ligase (C' and D'). The products of the reaction were analyzed as in Fig. 2. (A and B) Lanes 1, products of the ligation; lanes 2, digestions with BstNI (R1); lanes 3, digestions with RalI (R2); lanes 4, digestions with R1 + R2. (C, C', D, and D') Lanes 1, products of the ligation; lanes 2, digestion with (C and C') AvaII (R3), or (D and D') XmnI (R3); lanes 3, digestions with BstNI and the corresponding R3. (E) Two-dimensional gel analysis of the products from the experiment shown in A, lane 1. The scheme between panels C, D and C', D' indicates the origin of the R3 fragments seen in the autoradiographies. R1 to R3, and a to e as in Fig. 1.
ligation (Fig. 4B). The band seen at the monomer position in Fig. 4B (lanes 2 and 3) is not due to incomplete digestion by TaqI (cf. A and B). As it will be shown below (Fig. 6) these monomer fragments result from digestion of head-to-tail dimers. On the other hand, T4 DNA ligase accepts more readily the T/T mismatch, as shown by R3 (AccI) digestion, or by alkaline phosphatase treatment of the products of the ligation (Figure 4C). The relatively high blunt-end joining activity of T4 DNA ligase is emphasized by the production of covalently-closed circular (CCC) molecules containing the T/T mismatch (Fig. 4C, lanes 1 and 4 (CCC); the identity of the CCC molecules was verified by their change in electrophoretic mobility, relative to that of linear dimers, in neutral or alkaline gels (not shown), and the resistance of the label to alkaline phosphatase treatment (Fig. 4C, cf. lanes 1 and 4)).

When presented with equimolar amounts of perfectly matching AvaiI ends, both T4 DNA and chicken ligase join lesser proportions of mismatched, relative to matched, ends than in the case of the BstEII-ends (not shown). This indicates that the preference of both ligases to join perfectly matched cohesive ends depends primarily on the stability of the paired intermediate. However, in the absence of complementary intermediates the chicken ligase, and to a lesser extent the T4 DNA enzyme, accept rather efficiently non-complementary overhangs.

From the above results, we conclude that, in vitro, chicken ligase can produce mispaired molecules harboring a T/T or a A/A mismatch, 1-nucleotide away from the site of ligation. The efficiency of accepting the mismatches is not equal, the T/T being preferred to the A/A. T4 DNA ligase can only detectably produce the former under the same conditions.

The results also indicate that AvaiII, as opposed to BstEII (see above), is not able to cleave the mismatched sequences (Fig. 3 lanes 3). Since most restriction nucleases require a duplex substrate, the inactivity of AvaiII could also be due to the presence of nicks in the ligated dimers. However, analysis by 2-dimensional gel electrophoresis of the products of digestion (Fig. 4E) demonstrates that the mismatched termini were covalently joined on both strands (91% as measured by liquid scintillation counting). Hence, AvaiII cannot cleave the mispaired molecules at a significant rate.

The influence of the neighboring nucleotides on the ligation of mismatched-ends was examined by using the enzymes DdeI (TGA-) and HinfI (AGT-). The results obtained with chicken ligase are shown in Fig 5. As observed with AvaiII-ends (G(A/T)C-), significant numbers of dimers were produced. Restriction analyses indicate that ligated G/G mismatched ends
account for the main products of the reaction (Fig. 5, cf. lanes 3 and 4). In the case of the Ddel-Nael fragment, the persistence of the trimer band after Nael (R2) digestion (Fig. 5A, lanes 4 and 5) is due to partial cleavage by the enzyme, judging from the digestion of the indicator DNA (see Experimental Procedures).

Similar results were obtained with T4 DNA ligase. However, as already observed for AvaiI-ends, the proportion of blunt-end joining was almost as high as that of mismatched dimers (not shown). The data shown in Fig. 5 also demonstrate that neither DdeI nor Hinfl can cleave their mismatched recognition sequences at any significant rate.

Blunt to protruding-end joining.

The degree by which ligases might disregard sequence complementarity can be further explored by investigating the ligation products of DNA molecules containing 1-nucleotide long non-complementary ends. Fig. 6 shows the ligation of BstNI-Rsal fragments that have the potential for making a T/T or an A/A mismatch through the protruding BstNI-ends (panels A and B, respectively). Although these results were obtained with T4 DNA ligase, similar data were obtained with the chicken ligase (not shown, see Fig. 6, C' and D').

The ligase joins the monomer fragments into dimers and oligomers at significant rates, independently of the type of end (i.e. T-, or A-). As indicated above, although the dimers could be joined via the blunt-ends, the oligomers must represent ligation events also involving the BstNI-ends. However, the ligated fragments were completely refractory to BstNI digestion (Fig. 6A and 6B, lane 2), and only partially sensitive to Rsal (R2) digestion (lanes 3 and 4). The nature of the resistant products generated by T4 DNA ligase (Fig. 6, C and D) and the chicken ligase (Fig. 6, C' and D') was examined by digestion with R3 (AvaiI, Fig. 6, C and C'; XmnI, Fig. 6, D and D'). Surprisingly, none of the fragments predicted for molecules ligated through the BstNI-ends was observed (as indicated by the arrow d in Fig. 6, C and D). Instead, fragments longer than those expected by digestion of the monomers and blunt-end ligated dimers (fragments e and e' in Fig. 6, C, C', D, and D') were generated (Fig. 6, fragments of 1093-bp and 987-bp). In the case of AvaiI (R3) digestion, the labeled 987-bp fragment can only originate from head-to-tail ligation of a BstNI-end with a Rsal-end, while the two longer ones, 1093-bp and 1213-bp, are partials from incomplete digestion of monomers or head-to-tail dimers (see diagram in Fig. 6). This observation explains the resistance to Rsal (and BstNI) of the products of ligation shown in Fig. 6 (A
and B, lanes 3 and 4). Similar arguments can be used to demonstrate that a significant proportion of the dimers and oligomers produced by ligation of the 644-bp monomer reflect head-to-tail ligations (Fig. 6, D and D'). In this case, however, since XmnI (R3) cuts only once the monomer (producing 529-bp (labeled) and 115-bp (non-labeled) fragments), the head-to-tail molecules give rise to molecules of length indistinguishable from that of the non-ligated fragment a of 644-bp (Fig. 6, D and D').

If ligation of the head-to-tail molecules occurs through the protruding strands, it can be predicted that the joining event will produce gapped molecules. Two-dimensional gel electrophoresis of the ligated fragments suggests that this is, indeed, the case (Fig. 6E). Clearly, an important proportion of the dimers, and even more of the trimers and tetramers (cf. Figs. 4E and 6E) contain single strand discontinuities at the sites of ligation. A large proportion of gapped molecules is also observed by ligation of equimolar amounts of matching Bst.NI fragments (not shown), indicating that the rate of protruding to blunt end ligation is not negligible. This type of ligation is, of course, better seen when the protruding ends (complementary or not) are short because of the lower stability of the possible paired intermediates. However, it is also observed with longer non-complementary ends when the mispaired intermediates are not joined by the enzyme (Fig. 4B, lanes 2 and 3).

DISCUSSION

The main role of DNA ligases is the sealing of DNA nicks resulting from DNA synthesis. Although this reaction involves the joining of hydrogen-bonded substrates, the general ability of these enzymes to join short complementary-ends, and in particular that of the T4-induced enzyme to ligate blunt-ends is well known (19). The most important conclusion from our work is that DNA ligases can produce, rather efficiently, a variety of mismatched and gapped DNA molecules. Since our goal was more qualitative than quantitative, we have not attempted to optimize the conditions of the reaction. Thus, it may be possible to alter or increase the yield of the reaction products under certain conditions (e.g. ref. 20).

Our work has pointed to differences in the efficiency of T4 and chicken ligases in the joining of identical types of mispaired ends, the chicken erythrocyte ligase (or calf thymus ligase, unpublished observations) appearing as being more promiscuous. Results from mixing experiments suggest that this effect is not due to factors present in the partially purified enzyme...
preparations. It thus appears that the eukaryotic ligase is less affected by structural deformations of the helix near the site of action.

The presence of two independent DNA ligases in higher eukaryotes has been reported (3). DNA ligase I is the predominant activity in proliferating tissues, whereas DNA ligase II accounts for most of the activity in non-dividing cells. Since the immature erythrocytes from peripheral blood are arrested in G1 (i.e. less than 0.1% of S-phase cells as determined by flow cytometry, not shown) we suggest that the activity characterized is possibly that of DNA ligase II.

**Thermodynamic basis.**

The efficiency at which mismatched-end ligation occurs depends on a variety of conditions including concentration of enzyme and substrate, the particular type of mismatch, length and composition of the complementary sequence neighboring the mismatch, and origin of the enzyme. To compare the influence of the other parameters, we have worked with equivalent amounts of the enzymes and concentrations of DNA-ends.

As expected, the longer the complementary sequence between the mismatch and the site of ligation, the higher the yield of the reaction products. This is true both for T4-induced and chicken DNA ligases. In general, ends having G/G mismatches are joined better than those having C/C, and those having T/T better than those having A/A. Results from competition experiments and the inability of T4 ligase to produce A/A mispaired AvaII ends suggests that for a given length of overhanging-end, the efficiency of ligation follows the order G/G > T/T > A/A > C/C.

Measurements of thermodynamic parameters for synthetic oligonucleotides harboring all possible base-base mismatches have been recently reported (21, 22). Although the free energy of the duplex depends on the sequence surrounding the mismatch, these studies indicate that the order of stability for mismatches of identical bases is in agreement with our observations on the efficiency at which the ligases join the non-complementary ends. This correlation suggests that the rate of ligation is primarily influenced by the degree of interaction of the mispaired ends, even though none of the transient duplexes are likely to be stable under our experimental conditions (21, 22). In our study we have only examined a subset of all possible mismatches. However, since the relative stability of the C/C pair is the lowest of all the possible mismatches (21, 22), we would predict that the chicken ligase, and to a lesser extent the T4-induced enzyme, will be able to join all non-complementary ends, insofar as the mismatch is 1 to 2 nucleotides away.
from the site of ligation.

Although the relative rate of mismatch-end ligation depends on the stability of the paired intermediates, it is clear from our work that the actual product of ligation is simply dictated by the type of end available. This is most clearly seen in the reaction where ends containing non-complementary 1-nucleotide long overhangs were joined with blunt-ends in a head-to-tail fashion. This reaction also takes place when the ligases are presented with longer non-complementary ends. However, since in our experiments the concentration of ends is limiting, the relative amount of the longer ends available for head-to-tail joining is lowered through self-interaction and ligation.

Possible biological implications.

The in vitro properties of ligases make them potential candidates for introducing mutations in vivo during homologous and non-homologous recombination. Because of the lack of proof-reading mechanisms in these processes, mismatches introduced by ligases, as opposed to those due to mis-incorporation during DNA replication, could result in higher mutagenesis per event. The rate of fixation of the mutations may also be independent of the deformation introduced by the particular mismatch and of the ability of the repair systems to detect them (see refs. 12, 22-24).

Blunt-end ligation has been proposed to explain the products of non-homologous recombination of transfected DNA (25) and the joining of subgenomic DNA fragments (26). However, in the light of the in vitro properties of ligases, these events could be also explained by non-complementary head-to-tail ligations and subsequent filling of the gapped molecules. This mechanism can also easily explain the insertion of nucleotides of unknown origin at the junction of the recombined molecules. This might happen if a terminal-transferase activity would randomly add nucleotides to the protruding ends before the joining reaction takes place.

Fidelity of restriction nucleases.

Although there is extensive biological and experimental evidence that restriction nucleases are completely or largely inhibited from cleaving at mismatched sequences (e.g. 27, 28) our results clearly indicate that BatEII can easily cut sites containing a G/G or a C/C mismatch at an equivalent position. This contrasts with the behavior of AvaiI, DdeI, and Hinfl that do not tolerate mismatches at the position of the unspecified nucleotide.

In the case of AvaiI the T/T and A/A mismatches were tested, whereas in the cases of Hinfl and DdeI only the G/G mismatch was examined. From
thermodynamic considerations (22), two of the mismatches (G/G and A/A) have been proposed to form a wobble-pair structure, that is, to have a degree of order comparable to a regular base-pair. The other two (T/T and C/C) have been suggested to form open mismatches, that is, to show little interaction between the bases (22). Thus, it appears that the deformation in the duplex structure, even if minor, is sufficient to prevent productive interaction with these enzymes.

The fact that the mismatch is closer to the cleavage sites of AvaII, Hinfl, and Ddel, where a perfect fitting might be necessary for catalysis to occur (see ref. 27), could be a reason for the inactivity of these enzymes. However, it has been recently shown that HindII and TagI are able to cut at recognition sequences containing certain mismatches at the sites of cleavage (29). Hence, the ability of BstEII to cleave at heteroduplex sites suggests that its recognition sequence is made of two parts neighboring the unspecified nucleotide.

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