Temperature dependence of the joining by T4 DNA ligase of termini produced by type II restriction endonucleases

Luca Ferretti and Vittorio Sgaramella

Istituto di Genetica Biochimica ed Evoluzionistica del C.N.R., Via S.Epifanio, 14, 27100 Pavia, Italy

Received 8 October 1980

ABSTRACT

The temperature dependence of the T4 DNA ligase-catalyzed joining of plasmid DNA linearized by the action of HaeIII, EcoRI and PstI restriction endonucleases has been investigated by electron microscopy analysis. The extent of joining is maximal at 4° and decreases with increasing temperatures following sigmoid - like curves.

The temperature at which 50% of the maximal reaction is still observable increases going from DNA termini without single-stranded overlaps (produced by HaeIII) to termini with four nucleotides overlap, composed only by two A and two T (produced by EcoRI) to termini with four nucleotide overlap, composed by A, T, G and C (produced by PstI)

INTRODUCTION

The physiological substrate for the enzyme DNA ligase (1) is generally believed to be an interruption of a phosphodiester bond in a polynucleotide, with the resulting contiguous 5' P and 3' OH kept in register by an uninterrupted complementary DNA strand. A probably less natural but certainly very useful feature of the DNA ligase is the ability to join two different DNA duplexes, like those produced by most type II restriction endonucleases (2), when held together by hydrogen bonds between complementary sequences as short as two nucleotides (3). A peculiar feature of the T4 DNA ligase (4) is the ability to join end-to-end DNA duplexes possessing no single-stranded overlap, commonly called blunt or flush ended. The optimal temperature for the action of the DNA ligase on physiological

substrates is 37°, but when the enzyme is challenged with separate molecules, the stability of their interaction at the termini becomes also an important parameter in both rate and extent of joining. It has been found (5) that the melting temperature of the base-paired tetranucleotide (5'-AATT-3') termini produced by the EcoRI restriction endonuclease is 5-6°C, in low ionic strength buffer and 50% formamide.

For the covalent joining by the E.coli DNA ligase of cohesive termini produced by EcoRI Dugaiczky et al (6) have reported that in the 0-15° interval investigated, the optimal temperature was between 10 and 15°. Using heterogeneous populations of restriction fragments, Sgaramella and Ehrlich (7) found that for the cohesive termini produced by EcoRI, and also for the no-overlap, blunt termini produced by HaeIII, the maximal joining by the T4 DNA ligase was around 25°.

No study was known to us which dealt with the effect of the base composition of the complementary sequences on the temperature of joining of restriction endonuclease-generated termini. The data presented here describe the temperature dependence of the joining by the T4 DNA ligase, extracted from induced E.coli cells lysogenized with a lambda phage carrying the T4 gene for the DNA ligase (8,9) of the three different kinds of termini produced by the type II restriction endonucleases EcoRI, PstI and HaeIII (10).

MATERIALS AND METHODS

DNA. The plasmids pBR322 (11) and pC194 (12) have been isolated from clear lysates, (12,13), followed by CsCl-ethidium bromide equilibrium centrifugation.

Enzymes and proteins. The T4 DNA ligase has been extracted from thermally induced E.coli cells lysogenized with a recombinant phage harboring the T4 DNA ligase gene (8), kindly given to us by Drs. K and N.Murray. Details of the purifications procedure

are to be published. Restriction endonucleases: HaeIII was a gift of Dr.R.Roberts and used as described (14): PstI and EcoRI were from MRE, Porton Down, U.K., and used following the producer's instructions. T4 gene 32 product was a gift of Dr. H.Bujard.

Joining. The ligations have been performed under the following conditions: 20 µg/ml DNA; 50 mM Tris-Cl, pH 7.6; 5mM MgCl₂; 1 mM 2-mercaptoethanol; 500 µM ATP; 0.06 and 5 units of ligase per ml on cohesive and blunt ended substrates, respectively. After two hours at the indicated temperatures, the reactions were stopped by heating at 65° for 5 min.

Electron Microscopic (E.M.) analysis. Spreading was done in formamide (15). Picture were taken at a Philips EM 301 of randomly chosen fields. The contour lengths of circular and linear monomers and oligomers were projected, traced and measured with a manual map measurer. At least 200 molecules were blindly examined for each sample. The extent of ligation is the percentage of joined termini versus the total termini.

Agarose gel electrophoresis. Performed on an horizontal slab of 0.6% agarose in Tris-borate - EDTA buffer (pH 8.1).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. (SDS-PAGE) Performed essentially according to Laemmli (16).

RESULTS AND DISCUSSION

As substrates for our study, we have used the DNA of two plasmids, pBR322 and pC194, linearized with restriction endonucleases to generate molecules with the appropriate termini (see Fig. 1). Thus, pBR322, an E.coli plasmid of 2.9 Md (11), was cut to completion with (i) EcoRI to produce four-nucleotide single-stranded (cohesive) termini of sequence 5'-AATT-3', and (ii) PstI, to produce also four nucleotide cohesive termini of sequence 3'-ACGT-5'.

Flush-ends, i.e. termini without any single-stranded

Figure 1. Structures of the termini investigated in this study.

overlap, were produced by the action of HaeIII on the B.subtilis plasmid pC194 (12) of 2.0 Md. After E.M. control of the completion of the digestion, the restriction endonucleases were heat-inactivated (65° for 5 min) and the DNA substrates were diluted to 20 µg/ml.

A single reaction mixture was set up for each kind of termini, brought to the appropriate salts and cofactor concentrations (see Methods) and the enzyme was added. The mixtures were then split into aliquots which were incubated at the following temperatures: 4, 15, 25, 35 and 42°C. The ligations were allowed to proceed for two hours, when they were essentially complete.

The samples were then subjected to E.M. analysis (see Methods). Fig.2 gives the results of this study. The temperature dependence of the joining of the various substrates is characterized by sigmoid curves. All three kinds of termini, after 2 hrs of reaction at 4°, yielded extent of ligation close to 70%. At 15° only the blunt-ends show a noticeable decrease in joining, the two cohesive ends remaining unaffected. For all the three substrates, we have found almost parallel decreases of joining with increases in temperature up to 35°. At 42° only 5-10% of each termini can be joined. From these curves it is possible to derive a temperature value, $T_{\frac{1}{2}}$, at which half of the joining is still attained. The three termini exhibit clearly different $T_{\frac{1}{2}}$: under our experimental conditions (see Methods),

Figure 2. Temperature dependence of the joining of DNA termini produced by EcoRI ($\blacktriangle$), PstI ($\blacksquare$) and HaeIII ($\bullet$).

with HaeIII-produced termini, $T_{\frac{1}{2}}$ is the lowest, close to 23°; with the cohesive 4 A-T's termini produced by EcoRI, the $T_{\frac{1}{2}}$ appears to be close to 28°; finally with the 2 A-T's and G-C's termini produced by PstI, the highest $T_{\frac{1}{2}}$ is obtained, at 32°.

These results bear on a problem of great relevance to in vitro recombination: the joining of DNA termini produced by type II restriction endonucleases of common use like HaeIII, EcoRI and PstI.

Previous data (7) relative to the temperature dependence in the joining of heterogeneous segments produced by EcoRI and HaeIII suggested a bell-shaped dependence rather than a sigmoid one, as shown in this study. A possible explanation for this difference could be the interfering presence of contaminants of the ligase preparation used in that study, which were more abundant and most likely different from those of the present one. The enzyme used for those experiments had been extracted from E.coli cells infected with T4 phage, whereas the one used

in the present work comes from E.coli cells infected with a recombinant lambda phage harboring the T4 gene 30 only, that coding for the DNA ligase. From an SDS-PAGE analysis (Fig.3), it appears that this enzyme is much purer than the previously prepared one, and probably at least 60% physically homogeneous. It is conceivable that the contaminants in the old preparation might have reduced the extent of joining at low temperatures, for instance by binding to the substrates.

To clarify this possibility, to ligation mixtures containing EcoRI-pBR322 and HaeIII-pC194 as substrates, we have added as a "contaminant", a DNA binding protein, the product of T4 gene 32 (17). The ligated products have been analyzed by gel electrophoresis and electron microscopy. Fig.4 shows the gel pattern obtained with EcoRI-pBR322: it is apparent that at 4°C an amount of DNA binding protein equal in weight to that of the DNA has little or no effect on the extent of joining. A similar lack of effect has been noticed on HaeIII-pC194, analysed by electron microscopy (not shown).

Another possible explanation for the discrepancy between the earlier (7) and these data might be found in a stronger binding of contaminating proteins to the enzyme at the lower than at the higher temperatures, with a corresponding decrease of joining in the 5-15° interval. Whatever the reasons for the

Figure 3. SDS-Page analysis of T4 DNA ligase extracted from T4-infected E.coli cells (A) used in previous studies (7) and (B) of that used in this work and purified from induced lambda-lysogenized E.coli cells (8). In A were loaded approximately 500 μ g of protein, in B 25 μ g. Migration is from left to right: the numbers on the top derive from MW standards and are kilodaltons (Kd).

Figure 4. Lack of effect of T4 gene 32 product (DNA binding protein) on the joining of EcoRI-pBR322. A ligation mixture was set up, as described in Methods, containing an amount of gene 32 product equal in weight to DNA, and allowed to proceed for 2 hrs. The product was analysed by agarose gel electrophoresis. Lane 1: EcoRI-pBR322; lane 2: EcoRI-pBR322 exposed to a mixture of gene 32 product and T4 ligase; lane 3: EcoRI-pBR322 exposed to T4 ligase alone.

discrepancy between the old and the new data it seems reasonable to postulate that the properly apposed termini, ready to be ligated, must be more frequent at lower than at higher temperatures in the interval under study. It must also be emphasized that we allow the reactions to reach a plateau, so that increases in temperature elicit no increase in the extent of joining but either keep it constant or diminish it, when the substrate is destabilized and thus unavailable for joining.

The T_2^1 indicated in Fig.2 varied as expected qualitatively according to the nature of the termini involved in the joining. It is gratifying to note that also quantitatively the T_2^1 correlate well with the energies involved in the interactions of the three types of ends. In the case of the blunt ends produced by HaeIII, the relevant stacking forces can be estimated to be

close to 11.3 Kcal/mol (18). In the case of the cohesive termini, similar calculations give close to 21 Kcal for EcoRI-produced ends, and 34.6 Kcal for PstI-produced ends.

A practical consequence of the differences in $T_{\frac{1}{2}}$ could have been the possibility of uncoupling the joining of the blunt from the cohesive ends, or of two cohesive ends, differing in length and/or base composition. In the cases examined in this study, such an uncoupling is not possible, given the closeness of the three curves. In our laboratory other approaches are being investigated along this line, in view of the flexibility in mixed joining reactions obtainable if different termini could be joined in a controlled way by the same enzyme.

ACKNOWLEDGEMENTS

This research is in partial fulfillment of the doctorate thesis requirement of L.F. It has been supported also by a grant of Montedison SpA. We are indebted to Dr.G.Camerino for the SDS-PAGE characterization of the ligases. Ms F.Gallo Balma carefully typed the manuscript.

REFERENCES

1. Higgins,N.P. and Cozzarelli,N.R. (1979) Methods in Enzym. 68, 50-71.
2. Vosberg,H.P. (1977) Human Genet. 40, 1-72.
3. Miller,L.K. and Fried, M. (1976) Nature 259, 598-601.
4. Sgaramella,V. and Khorana,H.G. (1972) J.Mol.Biol. 72, 493-502.
5. Mertz,J. and Davis,R.W. (1972) Proc.Nat.Acad.Sci. 69, 3370-3374.
6. Dugaiczyk,A., Boyer,H.W. and Goodman,H.M. (1975) J.Mol. Biol. 96, 171-184.
7. Sgaramella,V. and Ehrlich,S.D. (1978) Eur.J.Bioch. 86, 531-537.
8. Murray,N.E., Bruce,S.A. and Murray,K. (1979) J.Mol.Biol. 132, 493-505.
9. Tait,R.C., Rodriguez,R.L. and West,R.W.,Jr. (1980) J.Biol. Chem. 255, 813-815.
10. Roberts,R. (1978) Gene 4, 183-193.

11. Hershfield,V., Boyer,H.W., Yanofsky,C., Lovett,M.A. and Helinsky,D.R. (1974) Proc.Nat.Acad.Sci. 71, 3455-3459.
12. Gryczan,T.J., Contente,S. and Dubnau,D. (1978) J.Bact. 134, 318-329.
13. Clewell,D.B. (1972) J.Bact. 110, 667-676.
14. Mottes,M., Morandi,C., Cremaschi,S. and Sgaramella,V. (1977) Nuc.Acid Res. 7, 2467-2475.
15. Davis,R.W., Simon,M. and Davidson,N. (1971) Methods in Enzym. 21, 413-428.
16. Laemmli,U.K. (1970) Nature 227, 680-685.
17. Bittner,M., Burke,R.L. and Alberts,B.M. (1979) J.Biol. Chem. 254, 9565-9572.
18. Bloomfield,V.A., Crothers,D.M. and Tinoco,J.,jr. (1974) in "Physical Chemistry Nucleic Acids" Harper and Row, page 72.