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Nonenzymatic Plasmid Ligation Mediated by Minor Groove-Binding Molecules

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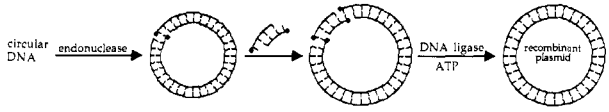
ABSTRACT: DNA ligation is the weak link in the chain of gene cloning. We have developed a straightforward nonenzymatic alternative to this reaction that employs easily available commercial reagents. The method uses the affinity of distamycin for the minor groove to join DNA ends together. Phosphodiester bonds are formed after cyanoimidazole-promoted phosphate activation in the presence of manganese(II) cations. When transfected into eukaryotic cells, the chemically ligated plasmid is transcribed even more efficiently than after enzymatic ligation. At present this technique compares favorably with T4 ligation for AT-rich cohesive termini, but in principle it could be extended to any restriction site.

The advent of gene technology in the early seventies was made possible by the discovery of enzymes such as site-specific restriction endonucleases and nonselective DNA ligases which are the cut-and-paste tools of recombinant DNA technology.



Chemists have developed many types of artificial endonucleases since that time [Schultz et al., 1982; Mitchell & Dervan, 1982; Schultz & Dervan, 1983; Barton & Raphaël, 1984; Nielsen et al., 1988; Constant et al., 1988; Bernadou et al., 1989; Behr, 1989; Saito et al., 1989; Baker et al., 1989; Corey et al., 1989; for reviews, see Barton (1985), Sigman (1990), Thuong and Helene (1993), and Knorre et al. (1994)], but useful hydrolytic reactions are scarce in the deoxy series (Corey et al., 1989; Pratviel et al., 1993) and true artificial DNases remain a challenge (Hendry & Sargeson, 1989; Jubian et al., 1992; Chin & Banaszczyk, 1989). However, triple helix-mediated sequence-specific cleavage is still useful for the sequencing of large eukaryotic genomes (Strobel et al., 1991). On the reverse side, ligation of DNA fragments into a vector is the weak link in the chain of gene cloning. This key step is usually catalyzed by bacteriophage T4 DNA ligase which links cohesive as well as blunt 3′-hydroxyl to 5′-phosphate termini together in the presence of ATP. From a chemical point of view, a phosphodiester bond is formed by nucleophilic attack of the ribose hydroxyl group on the AMP-activated 5′-terminus. Common sense suggests that construction of DNA is a harder task to achieve than its destruction. In recent years, however, several reagents more powerful than pyrophosphates (ATP, NAD) in activating phosphomonoesters in aqueous medium have been used to assemble nucleic acids in a double (Shabarova et al., 1981; Kanaya & Yanagawa, 1986; Dolinnaya et al., 1988, 1991a,b; Ashley & Kushlan, 1991; Kool, 1991; Pratviel et al., 1993) or triple (Luebke & Dervan, 1989, 1991, 1992) helix template. To be efficient, "chemical" ligation indeed requires the reacting ends to be in close proximity in order to minimize competitive reaction with the solvent. Oligonucleotides have therefore been ligated on large overlapping complementary sequences (the crystal structure of a nicked double-stranded oligonucleotide shows the potentially reacting groups to be very close; Ayamami et al., 1990), whereas plasmid DNA ligation requires additional homopurinic ends stuck together by an overlapping triplex-forming oligonucleotide. These approaches are especially interesting for the production of large amounts of oligonucleotides or for the synthesis of nucleic acids with nonnatural internucleotidic linkages which cannot be obtained enzymatically.

The major advantage of T4 DNA ligase is to join nanomolar concentrations of DNA ends issuing from virtually any restriction endonuclease, however, with highly variable success. Ligation often requires a large excess of enzyme and suboptimal reaction temperatures in order to stabilize dangling ends duplex formation, and tricks have to be found for blunt-end ligation (Sambrook et al., 1989; Rusche & Howard-Flanders, 1985; Pheiffer & Zimmerman, 1983). Even then, double-strand sealing is far from quantitative, and the resulting plasmid remains prone to endogeneous exonuclease digestion. The development of an efficient alternative to T4-mediated ligation would therefore be of real interest. In a previous paper, we have shown that a synthetic spermine–histamine conjugate acts as a ligase substitute in binding to DNA and enhancing the rate and yield of cyanoimidazole-promoted ligation of a nick (Zuber et al., 1993). Here we describe an alternative and more straightforward method for plasmid ligation that employs only commercially available chemicals. At present this technique works best with AT-rich cohesive termini, but in principle it could be extended to any sequence. Most importantly, when transfected into eukaryotic cells, the chemically ligated plasmid is transcribed even more efficiently than after enzymatic ligation.

MATERIALS AND METHODS

General. Ultraviolet spectra were measured on a Uvicon 930 spectrophotometer. Agarose gel quantitation was done with a Shimatzu CS-930 densitometer. Luciferase activity was monitored with a Berthold Biolumat LB 9500. Fast atom bombardment mass spectra (FAB MS) were recorded at the Laboratoire de Spectrométrie de Masse, Université Louis Pasteur. NMR spectra were recorded on a Bruker WP 200-SY spectrometer. For ^{31}P NMR data, chemical shifts were referred to the resonance of an external 85% phosphoric acid solution. All chemicals were reagent grade. Water was

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deionized on a Millipore Milli-Q apparatus. Metal ions were choride except for UO_2^{2+} and Cu^{2+} which were acetate. *N*-cyanoimidazole (ImCN) was prepared in a straightforward manner by mixing cyanogen bromide with imidazole in benzene, as described (Giesemann, 1955); it was stored at −20 °C in a dessicator. Purity was judged by melting point (59–60 °C) and by NMR spectroscopy in $CDCl_3$ (Kanaya & Yanagawa, 1986). ^{1}H NMR (D_2O) δ ppm: 6.9 (1 H, H-4), 7.4 (1 H, H-5), 8.1 (1 H, H-2). The lifetime of ImCN was estimated by monitoring the H-2 line decrease in 10 mM MES buffer, pD 6.0, with and without 20 mM $ZnCl_2$ at 20 °C. For activation of a model 5′-phosphate, 2 mL of a dGMP (free acid form, 35.6 mg, 100 μmol) solution in water was adjusted to pH 7.0 with 1 M NaOH. After lyophilization, the residue was dissolved in 2.5 mL of 0.2 M morpholinopropane sulfonate (MOPS)/NaOH buffer pD 7.0 in D_2O, and the ^{31}P NMR spectrum was recorded. ImCN (4.5 mg, 48 μmol) was added to the sample. After 3 h of reaction at 20 °C, the spectrum was recorded again. Guanosine 5′-phosphoroimidazolidate sodium salt (3.7 mg, 9 μmol) was then added to the mixture for comparison of chemical shifts. The genuine sample of deoxyguanosine 5′-monophosphoroimidazolidate sodium salt was prepared in 80% yield according to a published procedure (Mukaiyama & Hashimoto, 1971). ^{1}H NMR (D_2O) δ ppm: 2.2–2.5 (m, 1 H), 2.6–2.85 (m, 1 H), 3.85–3.95 (m, 2 H), 4.0–4.1 (m, 1 H), 4.4–4.5 (m, 1 H), 6.0 (t, 1 H), 6.75 (s, 1 H), 6.9 (s, 1 H), 7.6 (s, 1 H), 7.75 (s, 1 H). ^{31}P NMR (0.2 M MOPS buffer, pD 7.0) δ ppm: −8.05. FAB MS *m/z*: 442.0 (MNa^+), 420.1 (MH^+).

pBR322 plasmid was purchased from Boehringer-Mannheim. pGL2-control plasmid (Promega) was amplified in *Escherichia coli* and purified using standard column methodology. T4 DNA ligase was from BRL, and restriction endonucleases were from BRL and Boehringer-Mannheim. Enzymes were used according to the manufacturer's protocols. The linearized plasmids were extracted twice with phenol/chloroform, washed three times with chloroform, then with water-saturated diethyl ether, and precipitated with ethanol. The luciferase assay and microBSA kits were from Promega and Pierce, respectively.

Ligation Reactions. Most nonenzymatic reactions were performed at 20 °C during 16 h in a total volume of 20 μL containing 3.5 nM *Eco*RI-linearized pBR322 (0.2 μg), 10 mM MES buffer, pH 6.0, 50 μM distamycin, and 5 mM $MnCl_2$. Reaction was initiated by addition of an aliquot of a freshly prepared 50 mM ImCN solution in cold water to a final concentration of 5 mM. Reaction was stopped by addition of a 100 mM EDTA, pH 8.0, solution to a final concentration of 5 mM. DNA was then precipitated with ethanol. The residues were dissolved in 5 μL of water, mixed with 1 μL of a 30% glycerol bromophenol blue loading buffer, and subjected to electrophoresis in a 1% agarose gel containing 0.5 mg/L ethidium bromide, 1 mM EDTA, and 40 mM Tris/acetate, pH 8.0. DNA bands were visualized with a UV transilluminator, and uncorrected yields were estimated relative to the total signal of each lane by densitometric analysis of a negative photograph of the gel. Experiments with different reaction times, concentrations, restriction sites, metal cations, and DNA-binding molecules were carried out using essentially the same procedure.

For the enzymatic ligation reaction, the linearized pBR322 plasmid (0.2 μg, 3.5 nM) was incubated for 16 h at 20 °C with 1 Weiss unit of T4 DNA ligase in 20 μL of a solution containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and 5% PEG 8000. Reaction was stopped by addition of 100 mM EDTA, pH 8.0, to a final concentration of 10 mM. DNA was precipitated with ethanol and analyzed as described above.

Luciferase Gene Expression. *Eco*RI-linearized pGL2 (7 μg in 0.7 mL) was subjected to nonenzymatic ligation for 24 h at 20 °C as described above. For the enzymatic reaction, 40 T4 DNA ligase units were used. Reactions were stopped by addition of 0.5 M EDTA followed by ethanol precipitation. The DNA pellets were washed twice with 70% ethanol, dried, dissolved in water (1 mL), and quantified spectrophotometrically. Aliquots (0.2 μg) were withdrawn and analyzed by electrophoresis.

DNA ligation products of linear pGL2-control (2 μg) were transfected in triplicate with Transfectam into 3T3 murine fibroblasts (30 000 cells/35-mm dish) as previously described (Barthel et al., 1993). After 37 h, luciferase activity and overall protein concentration were monitored in the cell extract using the luciferase assay and microBSA kits.

RESULTS AND DISCUSSION

The development of a nonenzymatic alternative to T4 ligase requires both a reactant able to activate nanomolar phosphate concentrations in aqueous medium and a DNA-binding compound able to bring the reacting ends into close proximity without sequence specificity.

Three reactants meet the first requirement: carbodiimides, cyanogen bromide, and *N*-cyanoimidazole. The latter seems the most promising since water-soluble carbodiimides react slowly and are known to modify nucleic bases (Shabarova et al., 1981; Ashley & Kushlan, 1991; Dolinnaya et al., 1991b; Ehresmann et al., 1987); moreover, polyamine carbodiimides have been synthesized (Kiedrowski & Dörwald, 1988), but to our knowledge no chemical ligation has been reported with these compounds. Cyanogen bromide itself is inert. However, it may react with a buffer containing tertiary amine, giving an *N*-cyano tetrasubstituted ammonium salt as the reactive intermediate (Fodor et al., 1974; Paukstelis & Kim, 1974). Morpholinoethane sulfonate (MES) buffer has been shown to promote essentially unnatural 3′-phosphate end ligation (Dolinnaya et al., 1991a; Pratviel et al., 1993). In our hands, neither water-soluble carbodiimides nor BrCN led to DNA ligation with nanomolar concentrations of 5′-phosphate termini, although MES–, triethylamine–, and pyridine–BrCN adducts showed some 5′-phosphate oligonucleotide ligation at micromolar concentrations. Cyanoimidazole (ImCN) was first used for oligoribonucleotides ligation (Kanaya & Yanagawa, 1986; Ferris et al., 1989). It also may be generated in situ with BrCN/imidazole buffer and is efficient only in the presence of metal cations. ImCN/Zn^{2+} or Ni^{2+} mixtures (Luebke & Dervan, 1989; Kool, 1991) ligate oligodeoxyribonucleotides as well, and reasonable to good yields have been obtained for plasmid ligation reactions (Luebke & Dervan, 1991, 1992), hence our choice.

Second, a continuous double-helical shape at the sites to be ligated may be stabilized by ends overlapping with compounds that bind to DNA. Keeping a nonsequence selective ligation goal in mind, two alternatives may be envisaged: to target the distinctive structural or ionic features of the ligation site, or to saturate the whole plasmid with the drug, therefore including the site of interest. Intercalation between base pairs, although well known to display GC selectivity, could show some preference for the ligation site as well, since cationic aromatic compouds bind to structurally perturbated DNA bulges (Williams & Goldberg, 1988). Preliminary experiments were

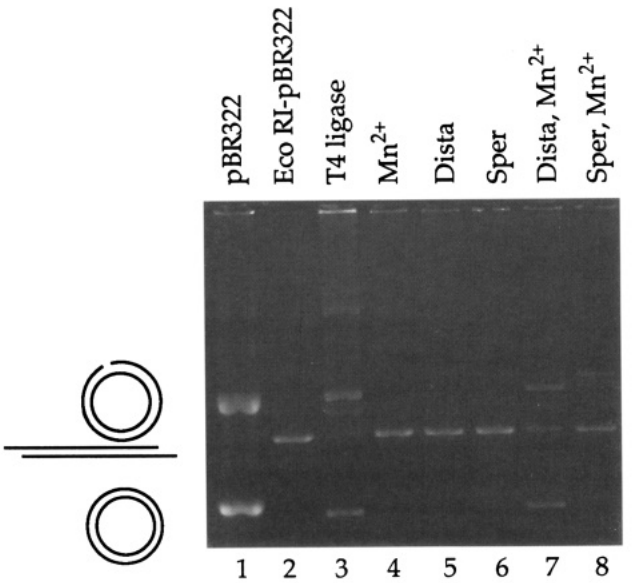

FIGURE 1: Analysis of the ligation products of *Eco*RI pBR322 DNA by electrophoresis through a 1% agarose gel containing ethidium bromide. Lane 1, untreated control pBR322; lane 2, *Eco*RI linearized plasmid; lane 3, ligation products of T4 DNA ligase (1 unit); lanes 4–8, *Eco*RI pBR322 DNA treated for 16 h at 20 °C with 5 mM ImCN in 10 mM MES buffer, pH 6.0, and 50 μM distamycin (lane 4); 5 mM $MnCl_2$ (lane 5); 200 μM spermine (lane 6); 50 μM distamycin and 5 mM $MnCl_2$ (lane 7); 200 μM spermine and 5 mM $MnCl_2$ (lane 8).

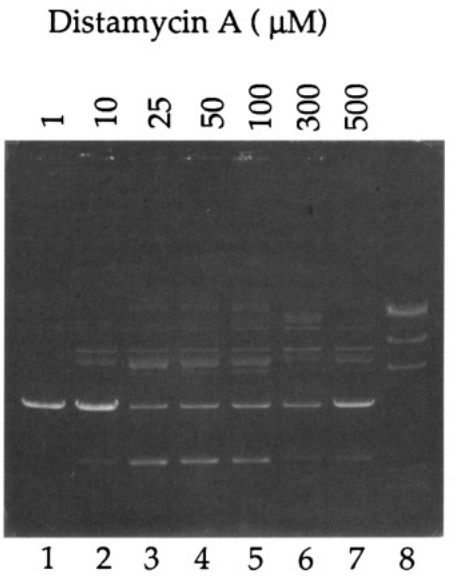

FIGURE 2: Effect of distamycin concentration on the yield of nonenzymatic ligation products. Reaction was performed for 16 h at 20 °C, with 3.5 nM *Eco*RI pBR322 (0.2 μg), 5 mM ImCN, 5 mM $MnCl_2$, and distamycin at the concentration indicated, in 10 mM MES, pH 6.0. Lane 1, *Hin*dIII–lambda DNA digest size marker; 1% agarose gel containing ethidium.

performed with 9-aminoacridine, which actually showed that oligonucleotide and plasmid ligation was inhibited by the drug, possibly because the reacting phosphate and hydroxyl ends are moved away upon intercalation. Major groove-binding molecules being strongly sequence-selective, we tested several cationic molecules which bind from the minor groove side. The general experimental frame was as follows: pBR322 has several unique restriction sites through which 4.3 kbp linear DNA molecules with various cohesive or blunt ends can be obtained. Plasmid ligation was performed in diluted conditions (3.5 nM) in order to favor the intramolecular reaction leading back to circular molecules. All compounds were separated after electrophoresis through an agarose gel containing ethidium bromide (Figure 1): single-strand ligation gives a circular nicked molecule which migrates slower than the starting linear one; when both strands are covalently closed, the plasmid is positively supercoiled by ethidium intercalation and migrates faster than the linear plasmid (and even slightly faster than the natural plasmid with initial negative supercoils).

Distamycin Efficiently Assists Nonenzymatic Ligation. *Eco*RI-linearized pBR322 has protuding 5′-pAATT ends. Distamycin A is a minor groove-binding oligopeptide antibiotic that shows distinct sequence specificity patterns [for review, see Zimmer and Wähnert (1986)] and has an affinity at least in the micromolar range for any stretch of at least four AT base pairs. Spermine is a natural polyamine which binds with K_D of ca. 100 μM in our conditions (Braunlin et al., 1982) to the minor groove of DNA with little sequence selectivity (Schmid & Behr, 1991). Thus the linear DNA molecule (3.5 nM; 15 μM bp) was reacted with ImCN (5 mM) in the presence of $MnCl_2$ (5 mM), distamycin (50 μM), or spermine (100 μM), in 10 mM MES buffer, pH 6, at 20 °C. After 16 h of reaction, the mixture was worked up (see Materials and Methods), and the DNA molecules were separated by electrophoresis (Figure 1). In the presence of distamycin (lane 7), the starting linear molecule (lane 2) was essentially converted to a slower and to a faster moving molecule, both of which comigrated with the major T4 ligation products (lane 3). These bands were respectively assigned to the single- and double-strand ligation products: The former comigrates with the nicked natural pBR322 (lane 1) whereas the other is spread into a ladder of several differently supercoiled topological isomers in the absence of ethidium (part of this latter topological isomer is actually ligated more than twice since some of the starting material was randomly nicked). All bands merge back into that of the linear molecule after *Eco*RI treatment, strongly suggesting that chemical ligation led to phosphodiester bonds. Spermine (lane 8) was less efficient and gave mostly a band corresponding to the length of a linear dimer. Polyamines and cobalt(III) hexammine indeed condense DNA into toroidal and rod-like structures containing several plasmid molecules (Bloomfield, 1991) which may favor intermolecular reaction. Spermidine as well as $Co(NH_3)_6^{3+}$ gave similar ligation profiles indeed (not shown). All ingredients ($MnCl_2$, ImCN, and spermine or distamycin) are required for the ligation reaction to occur (control lanes 4–6). Finally, the minor groove-binding dye Hoechst 33258 was also tested but gave no ligation products at all (not shown). Next, the concentration of distamycin was varied around that of the nucleic acid (15 μM bp). Figure 2 shows that 25–100 μM drug (lanes 3–5) lead to the highest ligation yield. Lower concentrations probably leave the ligation sites unfilled; higher concentrations may be unfavorable because of the formation of 2:1 distamycin–DNA site complexes (Pelton & Wemmer, 1990), in agreement with the occurrence of essentially single-strand ligation products (lanes 6 and 7) in which the minor groove may stay wider.

Ligation Mechanism and Yield. The time course of the ligation reaction (Figure 3) shows the double-strand ligation to be sequential: The yield of nicked circular plasmid quickly increases to a ca. 20% limit, whereas the circularly closed molecule appears with a lag time and becomes predominant after 6 h of reaction. After 12 h, which is three times the ImCN lifetime at room temperature ($t_{1/2}$ of ca. 4 h), the reaction has nearly stopped and reached a 75% overal yield of circular monomers (oligomers account to less than 5%). Further, to check whether the phosphomonoester sites

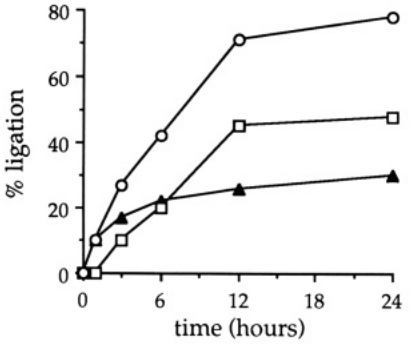

FIGURE 3: Time course of the ligation reaction. Aliquots containing 3.5 nM *Eco*RI pBR322 (0.2 μg, 15 μM bp), 5 mM ImCN, 5 mM $MnCl_2$, and 50 μM distamycin in 10 mM MES, pH 6.0, at 20 °C were stopped at various time intervals and electrophoresed on an agarose gel. Yields were determined by microdensitometry of negative photographs. Symbols: single-strand ligation product (filled triangles); double-strand ligation product (empty squares); total monomer ligation yield (empty circles).

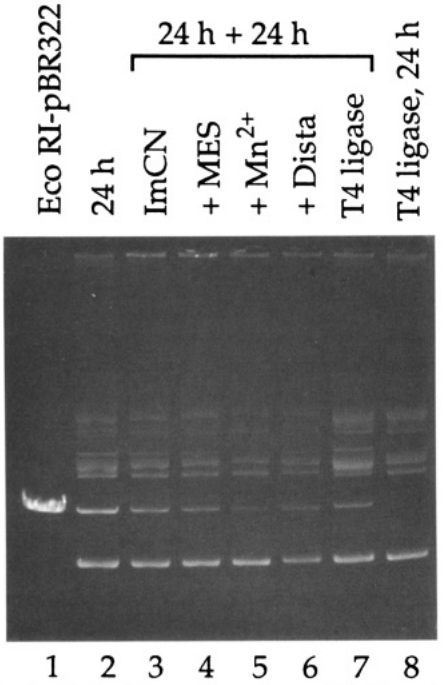

FIGURE 4: Analysis of the unligated material after a first nonenzymatic run. *Eco*RI pBR322 (0.3 μg) (lane 1) was ligated for 24 h with 1 unit of T4 DNA ligase (lane 8) or with 5 mM ImCN, 5 mM $MnCl_2$, and 50 μM distamycin in 30 μL of 10 mM MES, pH 6.0 (lane 2). After the first nonenzymatic run, ImCN (lane 3) plus the other reagents (indicated in lanes 4–6) were readded to the same concentrations as previously for a second run. (Lane 7) One unit of DNA ligase for the second 24 h run; 1% agarose gel containing ethidium.

remaining after a first run were converted to dead ends or not, all ingredients involved in the reaction were readded sequentially after 24 h. Figure 4 clearly shows that reaction had stopped because both ImCN and Mn^{2+} were consumed after the first run and that the double-strand ligation reaction goes now near to completion (91% of circular monomers, lane 5). This raises several questions about ImCN-activation of the 5′-phosphate and the role of the divalent metal cation. Phosphate monoesters may lead to reactive iminoimidazolides or imidazolides in the presence of ImCN. The occurrence of an intermediate in the ligation reaction has indeed been shown previously (Zuber et al., 1993). The nature of this intermediate was inferred from a model reaction involving 5′-GMP and ImCN (Figure 5): ^{31}P NMR shows a clean 10% conversion of 5′-GMP (δ = 3.30 ppm) into a compound with a chemical

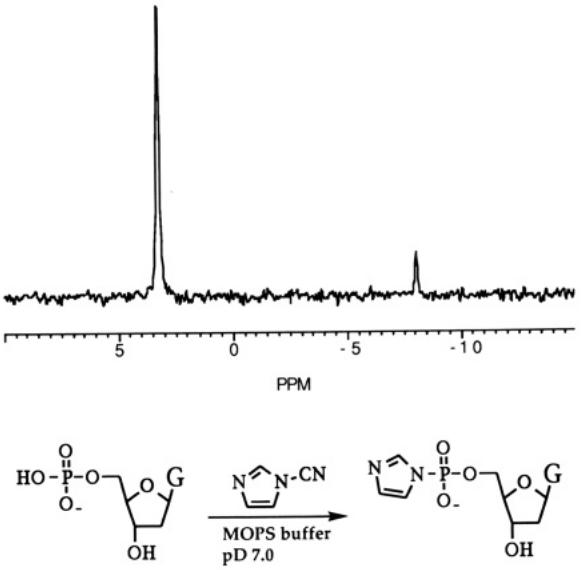

FIGURE 5: ^{31}P NMR spectrum of a mixture of dGMP (100 μmol) with ImCN (48 μmol) after 3 h at 20 °C in 0.2 M MOPS buffer, pD 7.0.

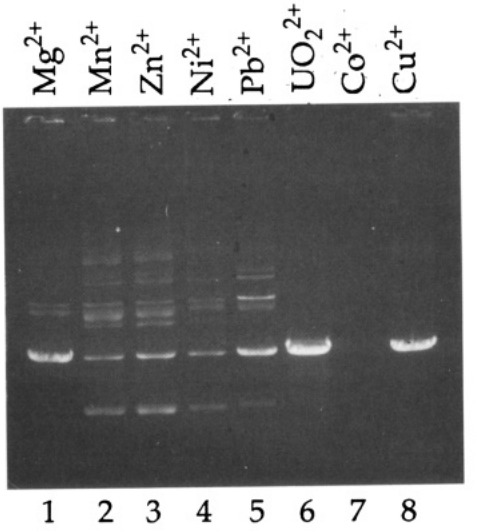

FIGURE 6: Effect of various divalent metal cations on the nonenzymatic ligation reaction. Mixtures containing 0.2 μg of *Eco*RI pBR322, 5 mM ImCN, 50 μM distamycin, and 5 mM of the indicated metal salt in 10 mM MES, pH 6.0, were left at 20 °C for 16 h. Electrophoresis was performed in a 1% agarose gel containing ethidium.

shift identical with that of an authentic sample of guanosine 5′-phosphoimidazolide (δ = –8.04 ppm). Similarly, ImCN has been shown to react with acetic acid in chloroform to give the *N*-acetylimidazole (Wentrup, 1972). Template polymerization of ribonucleotide 5′-phosphoimidazolides (Sawai & Orgel, 1975; Sawai, 1976; Roode & Orgel, 1980; Bridson & Orgel, 1980) as well as BrCN/Im-mediated ligation of ribonucleotides (Kanaya & Yanagawa, 1986) is enhanced by divalent metal cations. Various cations have been added to the linear pBR322 plasmid in the presence of ImCN (Figure 6 and Table 1), leading to the following order of efficiencies: Zn^{2+}, Mn^{2+}, $Ni^{2+} > Pb^{2+} > Mg^{2+} \gg Cu^{2+}$, UO_2^{2+}; Co^{2+} precipitates DNA. In the parent ribonucleotide series, zinc was shown to rather favor 3′–5′ bond formation (Bridson & Orgel, 1980), whereas the present low-efficient uranyl and lead cations were effective in the formation of 2′–5′ phosphodiesters from 5′-phosphoimidazolides (Lohrmann & Orgel, 1980; Sawai et al., 1981, 1989). These authors have extensively discussed the possible role of the metal cation in oligoribo-

Table 1: Ligation Yields Obtained with Various Divalent Metal Cations[a]

metal ion[b]	% coiled[c,d]	% total[c,d]
Mg^{2+}	0	19
Mn^{2+}	24	79
Zn^{2+}	29	79
Ni^{2+}	18	68
Pb^{2+}	6	52
UO_2^{2+}	0	0
Co^{2+}	–[e]	–
Cu^{2+}	0	0

[a] Experimental conditions are in the legend to Figure 7. [b] Chlorides, except for uranyl and copper (acetate). [c] Microdensitometry, uncorrected for ethidium differential binding to strained molecules. [d] Relative to the total signal for each lane. [e] Precipitates DNA.

nucleotide ligation; such development is beyond the scope of our work. Their conclusion points toward the nucleophily enhancement of the ribose hydroxyl, and since their order of efficiencies are essentially similar to ours, the same conclusions may hold here. However, nonproductive binding of the metal cation to ImCN hydrolysis products is an important practical aspect since a second addition of cation is necessary to bring the reaction to completion (Figure 4). Among Mn^{2+}, Zn^{2+}, and Ni^{2+}, manganese gave consistently higher yields and was used in all subsequent ligation reactions.

Chemical Ligation as an Alternative to T4 Ligase. In order to be of practical use for recombinant DNA technology, the nonenzymatic reaction conditions should not impair plasmid replication or transcription. Recent work (Shabarova et al., 1991; Luebke & Dervan, 1991) has shown that an artificially ligated plasmid remained able to transform *E. coli* (i.e., it is fully recognized by the bacterial DNA polymerase), without any mention of efficiency. To test this key point, we compared a chemically ligated plasmid to its enzymatically ligated counterpart for protein expression in an eukaryotic cell line (i.e., for full recognition by RNA polymerase II). pGL2, a 6.0 kbp plasmid encoding the firefly luciferase has a unique *Eco*RI site in the middle of the luciferase gene. This linearized plasmid was ligated with ImCN or T4 ligase under standard conditions (see Materials and Methods), and transfected with a lipopolyamine (Behr et al., 1989) into murine 3T3 fibroblasts. Luciferase activity was monitored in the cell extract 37 h later (Figure 7 and Table 2). The enzyme activity obtained from the luciferase gene-disrupted linear plasmid was 0.7% of the original pGL2 plasmid (incomplete *Eco*RI cleavage could account for the residual transcription level). Most importantly, ImCN/Mn^{2+} ligation restored activity (53%) to an even higher level than DNA ligase (17%), demonstrating that besides the productive ligation reaction, the 1650 base pairs of the luciferase gene remain essentially unaffected for the polymerase transcription process. All ligation experiments described above concerned *Eco*RI restriction sites. Minor groove-binding drugs have stronger van der Waals interactions with narrow groove regions. Accordingly, they show some preference for AT stretches without perturbation by any protuding guanine amino group. The aforementioned restriction site (G$^{\downarrow}$AATTC) falls into this favorable figure since the dangling p-AATT ends are stuck together by distamycin. *Hin*dIII restriction (A$^{\downarrow}$AGCTT) leads to protuding p-AGCT ends, and nonenzymatic ligation should be less efficient. However, the flanking 5′-AT and TAAT-3′ in the pBR322 sequence (Sutcliffe, 1979) may still provide drug binding sites and indeed an 80% ImCN-mediated ligation was observed in standard conditions. *Bam*HI (G$^{\downarrow}$GATCC) and *Sal*I (G$^{\downarrow}$TCGAC) restriction sites led to only ca. 15% and 1% ligation,

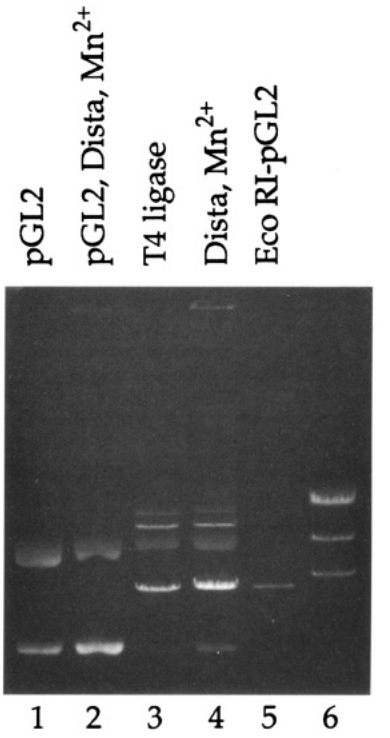

FIGURE 7: Analysis of the ligation products of *Eco*RI pGL2 DNA (7 μg) by electrophoresis through a 0.9% agarose gel containing ethidium bromide. Lane 1, untreated control pGL2; lane 3, ligation products of T4 DNA ligase (40 units); lane 4, *Eco*RI pGL2 DNA treated for 24 h at 20 °C with 5 mM ImCN, 5 mM $MnCl_2$, and 50 μM distamycin in 10 mM MES buffer, pH 6.0; lane 2, pGL2 treated as in line 4; lane 5, *Eco*RI linearized plasmid; lane 6, *Hin*dIII–lambda DNA digest size marker.

Table 2: Firefly Luciferase Gene Expression in Murine Fibroblasts

	luciferase activity ($\times 10^{-4}$)[a]	% of control (SD)
pGL2	1120 (177)	100 (–)
T4 ligation	192 (34)	17 (3)
nonenzymatic ligation	598 (82)	53 (7)
*Eco*RI pGL2	8 (1.4)	0.7 (0.2)

[a] Expressed as relative light units (10 s·mg of protein).

respectively (45% with T4 ligase). Blunt end ligation was deceiving even with the enzyme: *Eco*RV (GAT$^{\downarrow}$ATC; 3% yield with 5 mM ImCN, 100 μM spermine; 7% with T4 ligase).

In summary, for cohesive restriction sites providing a drug binding nest in the minor groove, the nonenzymatic ligation procedure described here provides an attractive alternative to the enzymatic one. The chemicals are comparatively cheap and indefinitely stable, and the reaction workup is straightforward (all compounds remain soluble in 70% ethanol where the plasmid is precipitated from). Ligation yields are similar with both techniques, yet the chemical approach seems to give more of the covalently closed molecule which in turn may explain its better performance in the transfection–expression experiment. Present limitations could be overcome with the search for stronger selective phosphate-activating reagents leading to short reaction times. On the other hand, improvements toward a general ligation technique may for instance be achieved with more extended oligopyrrole amides (Youngquist & Dervan, 1987; Griffin & Dervan, 1987) or mixed oligopyrrole–heterocyclic amides (Kissinger et al., 1987; Mrksich et al., 1992; Geierstanger et al., 1993; Mrksich & Dervan, 1993; Wade et al., 1993).

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