Nucleic Acids Research, 1999, Vol. 27, No. 3 875–881

High sequence fidelity in a non-enzymatic DNA autoligation reaction

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Received August 26, 1998; Revised and Accepted November 28, 1998

ABSTRACT

The success of oligonucleotide ligation assays in probing specific sequences of DNA arises in large part from high enzymatic selectivity against base mismatches at the ligation junction. We describe here a study of the effect of mismatches on a new non-enzymatic, reagent-free method for ligation of oligonucleotides. In this approach, two oligonucleotides bound at adjacent sites on a complementary strand undergo autoligation by displacement of a 5′-end iodide with a 3′-phosphorothioate group. The data show that this ligation proceeds somewhat more slowly than ligation by T4 ligase, but with substantial discrimination against single base mismatches both at either side of the junction and a few nucleotides away within one of the oligonucleotide binding sites. Selectivities of >100-fold against a single mismatch are observed in the latter case. Experiments at varied concentrations and temperatures are carried out both with the autoligation of two adjacent linear oligonucleotides and with intramolecular autoligation to yield circular 'padlock' DNAs. Application of optimized conditions to discrimination of an H-*ras* codon 12 point mutation is demonstrated with a single-stranded short DNA target.

INTRODUCTION

The ligation of DNA is an essential component of DNA repair, replication and recombination. One of the best characterized enzymes that join DNA ends is T4 DNA ligase, first isolated three decades ago (1–3). This and related proteins catalyze ATP-dependent phosphodiester bond formation between the 5′-phosphate and 3′-hydroxyl groups of adjacent DNA strands (4,5). Duplex DNA molecules with either cohesive ends or blunt ends can serve as ligation substrates (6,7). T4 DNA ligase can also repair nicked DNA duplexes efficiently and so the enzyme is often used in the laboratory for joining two DNA segments that are hybridized immediately adjacent to each other on a complementary strand.

When a single base pair mismatch exists at either side of the ligation junction, the efficiency of the enzyme to ligate the two oligodeoxynucleotides decreases markedly. This high sequence selectivity has resulted in the development of novel sequence detection methods using this enzyme. These approaches include the ligase detection reaction (LDR) (8–10) and the ligase amplification reaction (LAR) (11). T4 DNA ligase displays selectivities against single base mismatches on the order of 2- to 6-fold in yield and the presence of spermidine, high salt and low enzyme concentration have been reported to improve the fidelity of ligation to as high as 40- to 60-fold (9,10). The thermostable DNA ligase from *Thermus thermophilus*, *Tth* DNA ligase, has been reported to have higher fidelity; mismatch discrimination of 450- to 1500-fold in rate has been measured for the wild-type enzyme with optimized mismatch location at the 5′-side of the junction (12,13), making its use preferable in some sequence detection methods including ligase chain reaction (LCR) (14–16).

Since the discovery of ligase enzymes, there have also been developed a number of non-enzymatic approaches to joining the ends of two DNA strands (17–29). Non-enzymatic (chemical) methods for ligation may have some possible advantages over ligase enzymes in application to detection of mutations. Among these are lower sensitivity to non-natural DNA analog structures, the ability to be ligated on RNA targets, lower cost and greater robustness under varied conditions. In addition, non-enzymatic ligations might possibly be carried out inside intact cells or tissues which might not be accessible to ligase enzymes added to the medium. Despite these possible advantages, few of these chemical methods have been investigated for their sensitivity in detection of point mutations. Letsinger and co-workers reported ligation of short oligonucleotide fragments bearing electrophilic bromoacetamido and nucleophilic phosphorothioate groups and found that reaction yields were reduced 15-fold by the presence of a single nucleotide mismatch in a low salt buffer with elevated annealing temperature (19). Two photochemical ligation methods have also been investigated for mismatch discrimination (28,29). While the former did not present quantitative data, the latter reported that the ligation yield drops by a factor of five or more when a single mismatch is introduced at the junction. While non-enzymatic ligation methods may offer some advantages, one limitation of these chemical ligation strategies relative to enzymatic methods is the fact that the ligated structures differ from that of a natural DNA junction, which is likely to interfere with further manipulations (such as amplification) which are often used in sequence detection assays (30). The other disadvantage is that the reported selectivities are not as high as those for ligases.

A particularly promising approach to non-enzymatic ligation of DNAs is the reaction of phosphorothioates at the 3′-end of one strand with a leaving group (such as tosylate or iodide) on

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thymidine in the adjacent strand (31–36). The resulting joined DNAs differ from natural DNAs only by replacement of a single oxygen atom with sulfur. The method is also attractive because the DNAs containing these reactive groups can be prepared directly on automated synthesizers and are ligated without the need for added reagents (35). The product, a bridging 5′-phosphorothioester, is stable in solution and is resistant to enzymatic hydrolysis. Because of the close resemblance to natural DNA, the junction apparently does not affect the ability of polymerases to replicate or transcribe the sequence (36), which makes this ligation approach particularly promising for sequence detection methods that require further manipulation such as DNA amplification. However, as with enzymatic DNA ligation, this non-enzymatic method would be much more useful if its level of sequence selectivity were known.

We report here on studies aimed at delineating the sensitivity of this phosphorothioate–iodide DNA autoligation reaction to single nucleotide mismatches at or near the ligation junction. This is investigated in the context of H-*ras* target sequences both with dual probes ligating on the target DNA as well as for single probes designed to self-ligate intramolecularly to circular form (‘padlock’ probes; 37). The data show that optimized placement of a single base mismatch can lead to selectivities comparable with those seen with ligase enzymes.

MATERIALS AND METHODS

Preparation of autoligation probes

All oligodeoxynucleotides were synthesized on 1 µmol scale on an ABI model 392 synthesizer using standard β-cyanoethylphosphoramidite coupling chemistry. The 3′-end phosphorothioate groups required for the ligation reaction were incorporated into DNA strands as described previously (33,35). Briefly, the oligonucleotide synthesis was carried out with a 3′-phosphate controlled pore glass support (Cruachem). The first nucleotide unit was added with normal oxidation being replaced by a sulfurizing reagent (Applied Biosystems) (38). The remaining synthesis and deprotection were as for the standard DNA cycle. The second requirement for ligation is a 5′-end carrying an iodide. This is added with a commercially available 5-iodothymidine phosphoramidite reagent (Glen Research) (35). Deprotection and removal of iodine-containing strands from the CPG support was done by incubation in concentrated ammonia for 24 h at 23°C to avoid small amounts of degradation which occur at 50°C (35). Probe DNAs were then lyophilized and used without further purification, to avoid disulfide formation of the phosphorothioate ends. Analytical gels showed the purity of the phosphorothioate and iodide probes to be >90%.

Purification of target oligodeoxynucleotides was carried out by preparative denaturing polyacrylamide gel electrophoresis. All DNAs were quantitated by UV absorbance using the nearest neighbor approximation to calculate molar absorptivities.

Ligation reactions

Reactions were performed in 600 µl Tris–borate buffer, pH 7.0, containing 10 mM $MgCl_2$, with target and probe DNA concentrations of 1.3 or 20 µM. Ligations with radiolabeled probes also contained 50 µM dithiothreitol (DTT). Reactions were incubated at the indicated temperatures. Aliquots (100 µl) were removed at various times and then were frozen and lyophilized for 1 h. Pellets were taken up in 5 µl water/formamide/urea loading buffer.

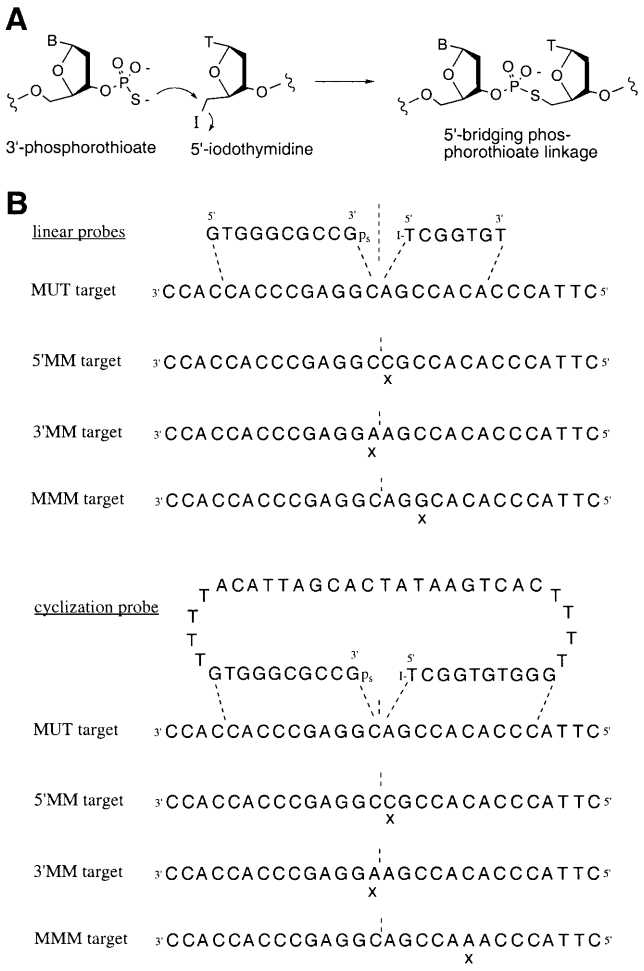

Figure 1. (**A**) Mechanism of iodothymidine-mediated autoligation, resulting in a 5′ bridging phosphorothioate linkage. (**B**) Sequences of autoligation probes and DNA targets. Mismatches between probes and targets are denoted by x. Target sequences are derived from H-*ras*; the 3′MM target corresponds to the protooncogene sequence and MUT to the codon 12 oncogenic mutation. The 3′-end phosphorothioate groups are denoted by p_S and iodothymine by I-T.

Samples were heated to 95°C for 2 min and then chilled on ice prior to loading on a 20% polyacrylamide gel containing 8 M urea. Gels were visualized with Stains-All dye (Sigma) and quantified by densitometry using NIH Image v.1.62b7 software. For radiolabeled probes, radioactivity was quantitated on a Molecular Dynamics PhosphorImager. The circular identity of the intramolecular probe after ligation was confirmed by isolation and treatment with S1 nuclease. This produced a second major band which co-migrated with the linear precursor.

RESULTS

Intermolecular autoligation

To evaluate the effects of complementary and mismatched template DNAs on the phosphorothioate–iodide autoligation reaction, we synthesized two probes 10 and 7 nt in length carrying a 3′-phosphorothioate and a 5′-iodothymidine, respectively. The sequences are given in Figure 1. We also synthesized four 28mer target DNAs which correspond to the fully complementary

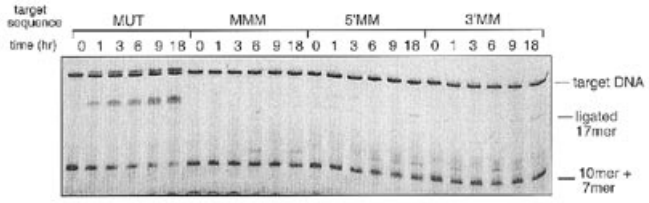

Figure 2. Ligation on varied target DNA sequences. Denaturing polyacrylamide gel electrophoretic analysis of autoligation of linear 7mer and 10mer probes on varied templates (sequences in Fig. 1). Bands were visualized by staining with Stains-All dye.

sequence (MUT) and singly mismatched targets where the position of the mismatch is at the 3′- and 5′-side of the junction (templates 3′MM and 5′MM, with G-A and T-C mismatches, respectively) and one in which a G-G mismatch is centered on the 7mer iodo-probe (template MMM, 'mid-mismatch'). The sequence of the fully complementary target corresponds to that of the codon 12 mutation commonly found in the H-*ras* oncogene (39), while the 3′MM target corresponds to the unmutated protooncogene sequence.

Ligations were carried out at pH 7.0 in a buffer containing 10 mM Mg^{2+}. We tested the effects of probe + target concentration and temperature on the extent and rate of ligation. The products of ligation were analyzed by following time courses over 24 h and were examined by denaturing gel electrophoresis (Fig. 2). Yields as a function of reaction time were quantitated by densitometry (Fig. 3).

Simple qualitative inspection of gels for a given set of reactions with these linear probes shows that both the reaction conditions and the relative placement of the mismatch are significant factors affecting the ligation yield. Figure 2 shows an example of a set of time courses with the four targets at 37°C and 1.3 µM probe and target concentration. The ligation proceeds to high conversion over 9–18 h with the complementary target (first six lanes), while only very little product is observed in the 3′MM and 5′MM cases and none at all in the MMM case. We also examined the same sets of ligations at 20°C with 1.3 µM DNA and at 37°C with 20 µM DNA (data not shown) and the results were qualitatively similar.

The quantitative comparisons of these three sets of conditions are shown in Figure 3. The data indicate that selectivity is highest at 37°C with 1.3 µM target and probe concentrations. In all three cases, the mid-mismatch (MMM) gives the highest level of discrimination; there is little difference between the junction mismatches (3′MM and 5′MM), which overall give lower levels of discrimination. At the two sets of conditions having lower DNA concentration there is no observable ligation on the MMM template at 24 h; we estimate that the densitometry could have detected 4% or higher yield at the last time point. This suggests selectivity of greater than one order of magnitude (below). In some of the slow ligation cases, a leveling off of the reaction appears to occur. In these cases it is possible that there is a small amount of disulfide formation between phosphorothioate probes that makes the kinetics appear biphasic. We have observed DTT-sensitive slower moving dimer bands when the probes are at high concentrations (such as during precipitation; data not shown). The quantitative kinetics experiments with radiolabeled probes were carried out with DTT present (below) to avoid possible disulfide formation.

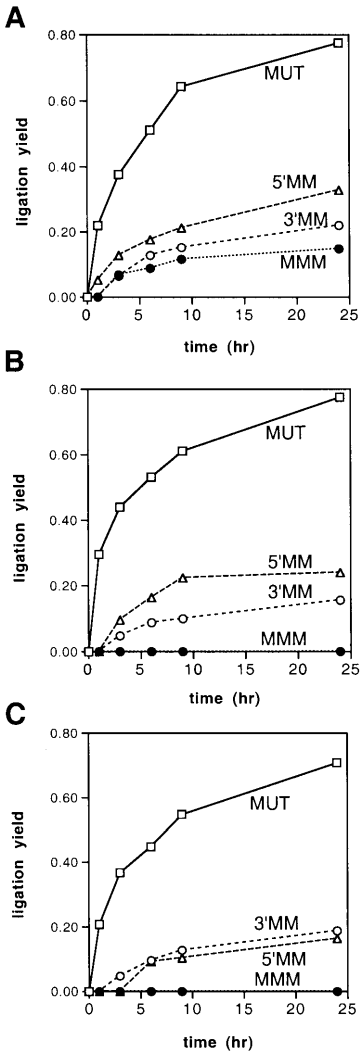

Figure 3. Effects of varied conditions on autoligation yields for 7mer + 10mer probes. (**A**) Time course of ligation at 25°C with 20 µM probe concentration. (**B**) Time course of ligation at 25°C with 1.3 µM probe concentration. (**C**) Time course at 37°C with 1.3 µM probe concentration.

Intramolecular autoligation/cyclization

We then investigated whether such ligations could be carried out intramolecularly to yield circular products. This type of ligation would be the non-enzymatic equivalent of those carried out with 'padlock'-type probes (30,37,39). Initially, the probe DNA was designed to form 10 bp on either side of the junction (sequences shown in Fig. 1). The reactions were again observed qualitatively, with analysis after 24 h (Fig. 4), and quantitatively, by following a time course under varied conditions (Fig. 5). Since the reaction is intramolecular we varied temperature (37 and 70°C) but not concentration.

The results show that the intramolecular phosphorothioate–iodide ligation proceeds in high yields with a fully complementary (MUT) target and that there is significant selectivity against single mismatches. In contrast to the intermolecular ligation, however, the levels of discrimination appear to be somewhat

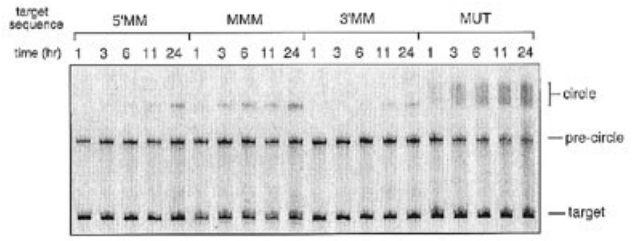

Figure 4. Ligation/cyclization on varied target DNA sequences. Denaturing polyacrylamide gel electrophoretic analysis of autoligation of 48mer 'padlock' probe on varied templates (sequences in Fig. 1). Bands were visualized by staining. Smearing of ligation product band is apparently due to secondary structure formed by this sequence and disappears when gels are run at high temperature (data not shown).

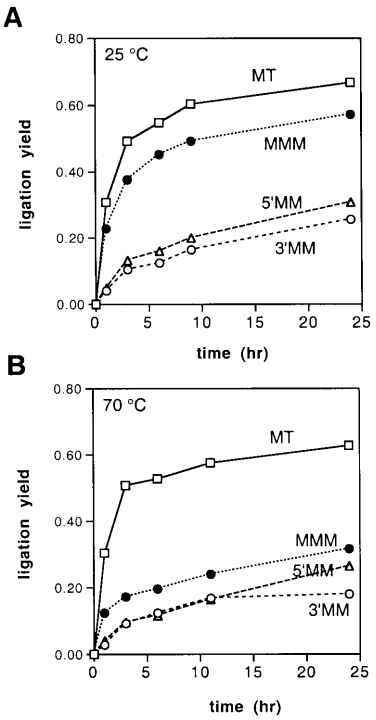

Figure 5. Effects of varied conditions on autoligation yields for 48mer cyclization probe having 10 nt of possible complementarity on either side of the junction. (**A**) Time course of ligation at 25°C with 1.3 µM probe concentration. (**B**) Time course at 70°C with 1.3 µM probe concentration.

lower. An example of the qualitative gel analysis is shown in Figure 4; here one sees significant amounts of ligation in all four cases. Smearing of the ligation product band in the presence of MUT target (last five lanes) is apparently due to interactions with the target during the running of the gel and disappears when gels are run at high temperature (data not shown). For the mismatches, the most ligation is seen with the central mismatch (MMM), which shows ~2-fold discrimination in yield at 24 h. Somewhat greater selectivity occurs with the 3′ and 5′ junction mismatches (3.5- and 2.5-fold). This is the reverse of what was observed for ligation of the intermolecular 7mer + 10mer probes, which showed highest discrimination in the MMM case. At 25°C with the cyclization probe, the MMM case shows only a very small

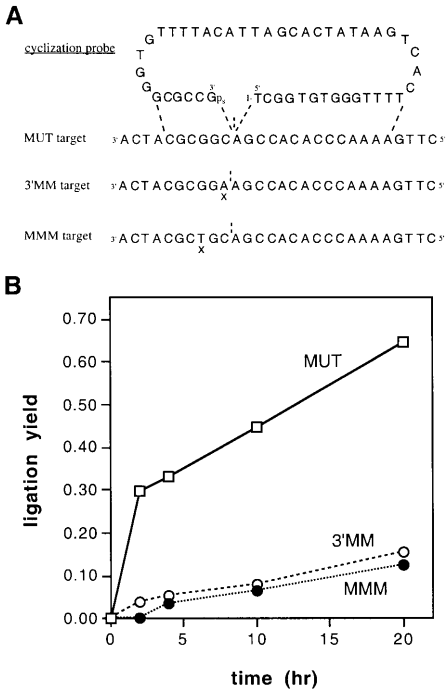

Figure 6. Effects of ligation junction placement on autoligation yields for 48mer cyclization probe having 6 and 14 nt of possible complementarity on the two sides of the junction. (**A**) Sequences of probe and target DNAs. (**B**) Time course of ligation at 70°C with 1.3 µM probe concentration.

amount of discrimination relative to the fully complementary sequence (Fig. 5A), while at 70°C the MMM discrimination increases somewhat (Fig. 5B). The magnitude of selectivity at 70°C in the 'padlock'-type case is similar to that seen for the 3′MM and 5′MM cases for the intermolecular reaction.

To test whether this ligation would be more sensitive to mismatches when located in a shorter binding domain, three new target DNAs were constructed (Fig. 6A). Binding of the cyclization probe to these new targets would give hybridization that still totals 20 bp, but arranged with 6 bp on one side of the junction and 14 bp on the other side. The MMM case has a mismatch that falls within the shorter 6 bp binding domain. Ligation experiments were again carried out with the three targets at 70°C. The data show (Fig. 6B) that, with this new sequence, selectivity against a mismatch at the junction is similar to that seen with 10 + 10 bp of hybridization. However, when the mismatch falls within the shorter binding domain, the selectivity is increased. At 20 h the comparative yields for the 6 + 14 bp case are 65% for the complementary (MUT) template and 13% for the mismatched (MMM) one. This compares favorably with the 10 + 10 bp case, which displayed yields of 63 (MUT) and 32% (MMM) at 24 h. It should be noted that the sequences are different in the 10 + 10 and 6 + 14 cases, however.

Optimized ligation of two probes for H-*ras*

The results indicated that in the intermolecular ligation, highest levels of discrimination would be observed with a mismatch located centrally in a short 7mer probe. To attempt to apply this optimized strategy to detection of the *ras* codon 12 mutation, we constructed two new probes (Fig. 7A) in which the well-

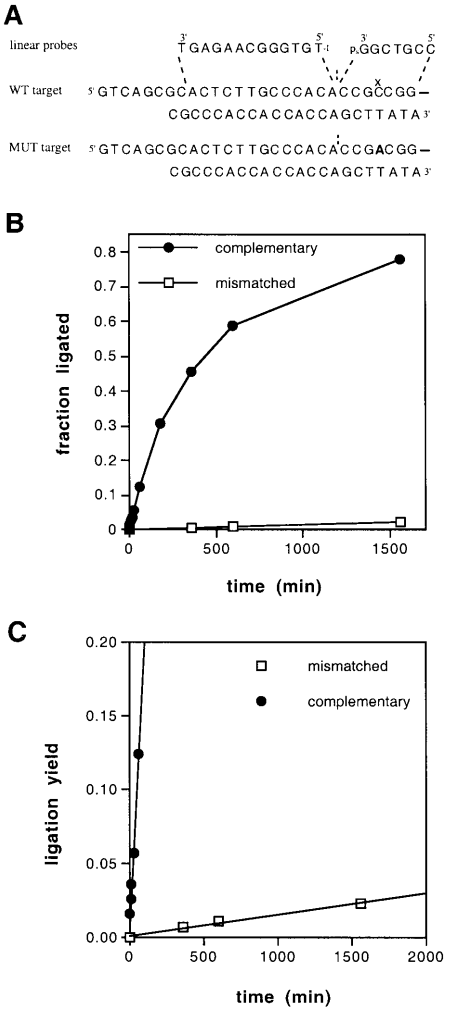

Figure 7. Optimized ligation of 7 and 13 nt probes on wild-type and codon 12 mutant H-*ras* DNA 50mer target strands, showing selectivity of probes for codon 12 mutant target. Ligation was monitored by use of a radiolabeled 7mer probe. (**A**) Sequences of H-*ras* optimized probes. (**B**) Time course of ligation at 37°C with 1.3 µM probe concentration. (**C**) Early time course showing initial rate linear fits. Correlation coefficients are 0.999 or better.

characterized G→T transversion (C→A in the complementary strand) could be targeted with the best mismatch location. We then evaluated the relative rates of ligation as described above, using the MUT 50mer complementary target (corresponding to the mutated oncogene) and the WT 50mer target (corresponding to the wild-type sequence). In this case the expected mismatch is a T-C located at the center of the 7mer phosphorothioate probe binding site.

The ligation was carried out at 37°C with 1.3 µM DNA and probes, conditions under which the earlier set of probes gave highest discrimination. The shorter phosphorothioate probe was 5′-radiolabeled to obtain greater sensitivity for quantitative rate measurements. The results are plotted as a time course in Figure 7B, which shows a quite large difference for the two targets. The data for the complementary ligation (MUT template) could be fitted well using a second-order kinetic fit (data not shown), giving a rate constant of 29.1 M^{-1} s^{-1}. The initial rates were measured for the two templates (Fig. 7C) and this allowed us to determine a rate constant of 0.16 M^{-1} s^{-1} for the same probes with the mismatched (WT) template. Thus, the rates are different by a factor of 1.8×10^2-fold, a level of discrimination comparable with that seen for ligase enzymes (below).

DISCUSSION

Utility of ligation reactions

The ability to detect single base differences in DNA is of great importance in molecular genetics. Specific identification of point mutations is playing an increasingly important role in diagnosis of hereditary disease and in identification of mutations within oncogenes, tumor suppressors genes and of mutations associated with drug resistance (40). Because of the high fidelity of ligation, enzymatic ligation methods have proven useful in a number of novel gene detection techniques (11–14,16). There are, however, a number of potential applications in which it would be useful to be able to join DNA strands without the need for ligase enzymes, as long as high fidelity in ligation could be maintained.

The present studies involving this non-enzymatic ligation are aimed primarily at quantitating and optimizing fidelity of the reaction and thus only single-stranded targets were used. It remains to be demonstrated how larger, more complex double-stranded DNA sequences might be addressed by this approach. Certainly, the problems presented by hybridization to double-stranded targets have been noted in studies of other sequence detection methods and a number of viable strategies for overcoming this have been described (10,41,42). Ongoing studies with this autoligation are examining a number of strategies for targeting one strand of DNA in the presence of its complement and preliminary studies have shown success with relatively long double-stranded sequences in slot-blot assays (Y.Xu and E.T.Kool, manuscript in preparation). In addition, ligations are also being carried out on RNA targets, which eliminates most of the problems presented by double-stranded targets.

At least with small single-stranded DNA targets, the present results show that the phosphorothioate–iodide autoligation reaction can proceed with good yields and high selectivities against single base mismatches, particularly when the mismatch falls near the center of a heptamer probe. In the optimized cases, G-G or C-T mismatches are selected against by a factor of at least two orders of magnitude. This level of specificity is higher than that seen for the same mismatches using phage T4 DNA ligase (8–10) or *Chlorella* virus DNA ligase (43), although it is not as high as that seen for the more discriminating bacterial *Tth* ligase under optimized conditions (15). Although it remains to be seen whether this level of discrimination will generally hold true for other mismatches in autoligation, the results do indicate that this method shows significant promise in probing the H-*ras* point mutation and merits further study with other sequences. In addition, application of this autoligation to current assay methods such as LDA and ligation-mediated PCR should be examined.

Mechanism of ligation selectivity

It is of interest to consider the physicochemical origins of the selectivity for this autoligation reaction. The data show that selectivity is much lower at the ligation junctions but is high near the center of a short probe. We surmise, therefore, that the chief factor in successful ligation is the binding affinity of the probe rather than the precise geometry at the ligation junction. This is consistent with our finding that the reaction apparently proceeds

with second-order kinetics. Enzymatic ligations are commonly most selective at the junction, a fact which is attributed to the precise geometric control that the enzyme takes in orienting the reactive groups for in-line attack at phosphorus (5). For the present reaction it seems that the transition state S_N2 geometry can be reached even with mismatched geometries; this is likely due to the relatively high flexibility of the DNA at the nicked junction. Presumably, ligase enzymes curtail this mobility to a high degree.

Our results suggest that the intermolecular ligations occur with higher levels of mismatch discrimination than do the intramolecular cyclization cases examined (Figs 3 and 6). Before this can be firmly concluded, additional studies with identical sequences will be needed. However, if it is the case, then this is consistent with a higher effective concentration of the reactive groups relative to one another, due to their being linked. We hypothesize that mismatched cases benefit from this linkage to a greater extent. Successful intermolecular ligation requires strong binding and thus long residence times of the two reactive groups near one another; in the intramolecular case the two reactive ends are never far from one another even if one end is not bound to the target DNA. Interestingly, such effects might possibly be operative in enzymatic ligations as well. It would therefore be of significant interest to compare enzymatic ligation fidelities for inter- and intramolecular cases in the same sequences. Although intramolecular 'padlock' probes have been examined in a number of laboratories recently (30,37,39), we know of no study in which ligation fidelity has been quantitated for such probes.

Comparison of autoligation and other chemical ligation methods

A number of other non-enzymatic strategies can also be used in a template-directed fashion to ligate two DNA probes (17–29). Of these, few have been quantitatively examined for ligation fidelity and so most cannot yet be compared with the current approach. The ligation of a phosphorothioate with a bromoacetamide group and photochemical ligation via thymine dimers are two methods that do show significant selectivity, in that yields are lowered in the presence of a mismatch (19,28,29). Comparative rate data with and without mismatches are not yet available for those reactions.

One significant difference between most chemical ligations (17–29) and autoligation approaches (32–36) is that autoligation requires no reagents to be added for it to proceed. Other chemical ligations require reducing reagents such as borohydride (20), oxidizing reagents such as ferricyanide (18), condensing reagents such as carbodiimides or cyanoimidazole (21,22,24–26) or UV irradiation (28,29) to carry out the reaction. Although the need for added reagents is not limiting in many situations, autoligation is simpler and might be carried out in media where reagents are inactive or where they will affect biochemical processes.

Another distinction between the present autoligation and most non-enzymatic approaches to DNA ligation is that this autoligation, unlike other methods, produces a structure closely resembling that of natural DNA. We have shown that the 5′ bridging phosphorothioate (5′-S) junction produces little or no thermal destabilization of duplexes (36) and that it is stable to hydrolysis over a wide pH range. Moreover, DNA strands containing the bridging phosphorothioate can act as normal templates for DNA and RNA synthesis (36), which is not likely to be the case for many other ligation chemistries. While 5′-S junctions inhibit some nuclease enzymes, they can be chemically cleaved by the use of aqueous silver ions (44).

Comparison of autoligation and enzymatic ligation

It remains to be seen whether the phosphorothioate–iodide autoligation reaction might be used in many applications to replace enzymatic ligations or whether it will be more useful in a limited set of specific cases. It is worth noting that this autoligation has two limitations which might affect its general utility. First, since the iodide exists only on a thymidine residue, there is at present a sequence limitation in which an adenine must be present at the desired ligation site. If either sense or antisense strands of the DNA target can be probed, then the restriction is relaxed to either T or A at the junction, which means that at least half of all possible sites can be probed optimally. Since in most applications the ligation junction can be shifted by 1 or 2 nt as needed, this is not expected to be a major problem. Nonetheless, some potential targets might not be optimally probed, especially in G,C-rich runs. It is possible that this could be remedied by synthesis of an analogous iododeoxycytidine nucleoside derivative, but such a compound is not currently available. A second possible limitation of the phosphorothioate–iodide ligation is that it requires longer times than enzymatic ligations. In one comparison we noted that the autoligation proceeded approximately three or four times more slowly (36); thus, while an enzymatic ligation might be nearly complete in 3–6 h, the present reaction requires ~12–18 h. While for many applications this difference may not be important, in some high-throughput assays it may be a significant factor.

On the other hand, there are a number of aspects of the autoligation reaction which may give it advantages over standard enzymatic approaches. Since no enzymes or added reagents are needed, the ligation might be carried out in media that would prevent enzymatic reactions. For example, it is conceivable that the ligation could be carried out inside whole cells or tissue samples or in gels, solvents or physical conditions not amenable to enzyme permeability or stability. Since the ligation proceeds well in the presence of millimolar amounts of DTT (Y.Xu and E.T.Kool, work in progress), it is feasible that the reducing environment of the cell might not interfere with reactivity. Another benefit of the autoligation is that it can be used with short probes such as heptamers, which cannot be ligated by the commonly used *Tth* ligase (45). In addition, the absence of enzyme requirements makes it feasible to carry out ligations of structures which are not substrates for DNA ligases. Examples of this might be the ligation of DNA probes on RNA targets and the use of chemically modified DNA probes. A further benefit of this autoligation reaction relative to enzymatic methods is that the 3′ probe cannot serve as a primer for polymerases, since it has a phosphorothioate group blocking the 3′-hydroxyl. In standard enzymatically ligated probes, both unligated probes as well as the product can potentially act as primers. It is worth pointing out that since the phosphorothioate and iodide probes are constructed on an automated synthesizer using commercially avaliable reagents, requiring no post-synthesis modification, these potential benefits can be realized without any additional preparative effort over standard methods.

ACKNOWLEDGEMENTS

We thank the National Institutes of Health (GM46625) and the Army Research Office for support.

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