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Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases

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ABSTRACT

DNA polymerases catalyze the addition of deoxyribonucleotides onto DNA primers in a template-directed manner. The requirement for template instruction distinguishes these enzymes from other nucleotidyl transferases, such as terminal deoxynucleotidyl transferase, that do not utilize a template. An oligonucleotide substrate was used to characterize a novel, non-templated nucleotide addition reaction carried out by DNA polymerases from a variety of procaryotic and eucaryotic sources. The products of the reaction, in which a deoxyribonucleotide was added to the 3' hydroxyl terminus of a blunt-ended DNA substrate, were analyzed by electrophoresis on high resolution, denaturing polyacrylamide gels. DNA polymerase from Thermus aquaticus, polymerase α from chick embryo, rat polymerase ß, reverse transcriptase from avian myeloblastosis virus, and DNA polymerase I from Saccharomyces cerevisiae all carried out the blunt-end addition reaction. The reaction required a duplex DNA substrate but did not require coding information from the template strand. These results demonstrate that template instruction is not an absolute requirement for the catalysis of nucleotidyl transfer reactions by DNA polymerases.

INTRODUCTION

The requirement for faithful transmission of genetic information from parent to progeny has resulted in the evolution of highly accurate enzymatic mechanisms for replicating DNA (1). Estimates for the frequency of base substitution errors during DNA replication in vivo range from 10^{-7} -10^{-11} misincorporations per base pair replicated (2-4). Error frequencies during in vitro DNA synthesis have been determined for a number of DNA polymerases (for reviews see 5,6). Moreover, the polymerase errors produced during DNA synthesis in vitro have been shown by sequence analysis to consist largely of base substitutions and single-base frameshifts (7,8).

The precise structural features of the DNA substrate that are recognized and used by a polymerase to discriminate between correct and incorrect insertion events are not well understood at present. Formation of the phosphodiester bond between the incoming dNTP and the 3' hydroxyl terminus of the primer normally requires that a proper base pair be formed between the base moiety of the dNTP and the appropriate base in the template (1). However, non-templated nucleotide addition events occur during the rearrangement of immunoglobulin genes (9) and may also be involved in the replication of the telomeres of eucaryotic chromosomes (10,11). The enzymes that catalyze these non-templated events in vivo have not been identified with certainty, although the involvement of terminal deoxynucleotidyl transferase (12) in immunoglobulin rearrangement has been suggested (9). A terminal transferase-like activity from Tetrahymena thermophila that

catalyzes the addition of telomeric DNA sequences to a single-stranded oligonucleotide primer has also been described (13). Recently we reported that the large fragment of DNA polymerase I of E. coli has the ability to add one or more nucleotides onto the 3' terminus of a blunt-ended DNA substrate in a non-templated fashion (14). The novel nature of this DNA polymerase-mediated reaction prompted us to screen a number of DNA polymerases for this capability. We now report that the ability to carry out non-templated nucleotide addition is a property shared by DNA polymerases from both eucaryotic and procaryotic organisms.

MATERIALS AND METHODS

Reagents

Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from International Biotechnologies, Inc., New Haven, CT. DNA polymerase from the thermophilic bacterium Thermus aquaticus (15), referred to as Taq DNA polymerase, was obtained from New England Biolabs. Recombinant rat DNA polymerase ß was obtained from Samuel Wilson (National Institutes of Health, Bethesda, MD). DNA polymerase α from chick embryo, fraction VII (16), was provided by Akio Matsukage, Aichi Cancer Center Research Institute, Nagoya 464, Japan. DNA polymerase I from Saccharomyces cerevisiae, purified either as described (17) or by immunoaffinity chromatography, was provided by Akio Sugino and Robert Hamatake of this institute. Polymerase α and yeast DNA polymerase I prepared by conventional methods (17) have not been purified to homogeneity; the immunoaffinity-purified yeast polymerase I has been purifed to homogeneity. Nucleotide precursors, HPLC purified, were obtained from Pharmacia. Oligonucleotides were synthesized and purified as described (18).

Preparation of DNA substrates

The blunt-ended duplex was formed by annealing two complementary pentadecanucleotides, one of which had been labeled with ^{32}P at its 5' end. The substrate used for determining the relative rates of templated versus non-templated synthesis consisted of a 5'-labeled 14mer annealed to an 18mer template. The labeling and annealing reactions were carried out essentially as described (18), with the unlabeled strand present in the annealing mix in a two-fold excess over the labeled strand. The DNA concentration was approximately 2.5 ug/ml for the blunt-end duplex and 3.5 ug/ml for the 14mer/18mer substrate.

DNA synthesis assays

Each blunt-end duplex reaction contained approximately 2.5 ng of DNA, either 400 uM each of all four dNTPs or a single dNTP at 400 uM, and 0.15- 1.8 units of polymerase (see Figure legends). The enzyme units for DNA polymerase ß, Taq DNA polymerase, and AMV reverse transcriptase are those of the supplier. Enzyme activities for polymerase α and yeast polymerase I were determined by Robert Hamatake on an activated DNA substrate as described (17). The blunt-end addition assays were carried out in a volume of 5 ul for 60 minutes at 37 °C; Taq polymerase reactions were carried out at 55 °C for 30 minutes. AMV reverse transcriptase reactions were

carried out in the buffer described by Maniatis et al. (19). Rat polymerase ß and yeast polymerase I reactions contained 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol. The buffer for polymerase α was the same as that for polymerase ß except that the pH was 7.4. The buffer for Taq polymerase contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.1 % gelatin.

Rate determination reactions were carried out at 37 °C in a volume of 40 ul containing approximately 20 ng of the 14mer/18mer substrate, 400 uM each of all four dNTPs, and 8-28 units of enzyme (see figure legends). The buffer conditions were as described above. At the appropriate times, 5 ul aliquots were removed and prepared for electrophoresis. All reactions were terminated by the addition of a dye/formamide solution and aliquots were analyzed by electrophoresis on 20 % denaturing, polyacrylamide gels as described (20). Autoradiography was performed at -20 °C for 1-3 hours with an intensifying screen.

RESULTS

Non-templated addition by Taq DNA polymerase

The substrate that was used to monitor the blunt-end addition reaction consisted of two complementary pentadecanucleotides annealed to form the following blunt-ended duplex:

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5' 32P-GTCCGTCTCTGCCTC   3'
3'     CAGGCAGAGACGGAG   5'
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The unlabeled "template" strand of the duplex contains no thymine residues and cannot provide coding information for the addition of dATP. Therefore any addition of dATP to the 3' hydroxyl terminus of the labeled primer must represent a non-templated event. This substrate was used in our earlier experiments to verify that the preferential addition of dATP to a blunt end by DNA polymerase I (Klenow fragment) of E. coli occurred via a non-templated pathway (14). The products of the blunt-end addition reactions were monitored by electrophoresis on high resolution, polyacrylamide gels. Figure 1 shows the results of an experiment in which DNA polymerase from the thermophilic bacterium Thermus aquaticus was incubated with the blunt-end duplex in the presence of equimolar concentrations of all four dNTPs or with individual dNTPs. Taq DNA polymerase used any one of the four dNTPs to carry out the blunt end addition reaction when the precursor was supplied individually (Figure 1, Lanes A,T,C,G). However, dATP was used much more efficiently than the other dNTPs. The electrophoretic mobility of the +1 product varied slightly depending upon which nucleotide was added to the 3' end of the primer strand, thus providing a simple means of identifying which nucleotide was added when all four were present (14). For example, oligonucleotides terminating with a dCMP residue (Figure 1, Lane C) run slightly ahead of those that terminate with dAMP (Figure 1, Lane A). Taq DNA polymerase showed a strong preference for the addition of dATP when all four dNTPs were included in the reaction (Figure 1, Lane 3). We base this conclusion on the fact that the electrophoretic mobility of the +1 addition product synthesized in the presence of all four dNTPs is identical to that of the product synthesized

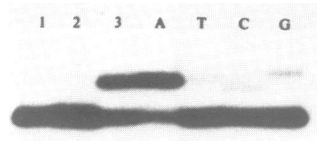

Figure 1. Electrophoretic analysis of non-template directed, blunt-end addition products synthesized by Taq DNA polymerase on duplex DNA having a flush end. The sequence of the DNA substrate is given in the text. Lane 1 is the unextended 15mer marker. Lane 2 shows the product of a reaction carried out with the single-stranded primer in the presence of all four dNTPs. The remaining lanes show the products of reactions carried out on the duplex substrate with all four dNTPs (Lane 3), dATP (Lane A), dTTP (Lane T), dCTP (Lane C), or dGTP (Lane G). Each reaction contained 0.15 unit of Taq DNA polymerase.

with dATP alone. No addition to the single-stranded primer was observed (Figure 1, Lane 2), indicating that a duplex DNA substrate was required for +1 addition to take place.

Non-templated addition by mammalian DNA polymerases

Experiments similar to those described above were carried out with chick embryo polymerase α and with rat polymerase ß (Figure 2). Both polymerases were able to catalyze non-templated nucleotide addition. For polymerase α, the yield of +1 product was considerably greater when purine nucleotides, particularly dATP, were provided individually (top portion of Figure 2, Lanes A,T,C,G). Moreover, in the presence of all four dNTPs, the +1 product synthesized by polymerase α terminated in a 3' dAMP residue (Figure 2, top; Lane 2). A significant fraction of the starting 15mer population was also converted to 14mers and smaller fragments, probably as a result of trace nuclease contamination in this preparation of polymerase α. It is not clear whether the apparent bias towards the use of dATP reflects the intrinsic specificity of nucleotide addition by polymerase α or preferential exonucleolytic degradation of +1 products that terminated with nucleotides other than dAMP. Polymerase ß showed a similar bias towards the use of dATP when all four dNTPs were present in the reaction (bottom portion of Figure 2, Lanes 2 and 3). In the presence of all four dNTPs, exonuclease activity (as judged by the amount of 14mer formed) was negligible compared to the amount of +1 product formed. Therefore, the bias for addition of dATP appears to be an intrinsic property of polymerase β. However, in contrast to polymerase α, the yield of +1 addition product was higher with dCTP than with dGTP when the dNTPs were supplied individually. Neither enzyme was able to add nucleotides onto the single-stranded primer (data not shown). Surprisingly, significant amounts of the 14mer were also produced by polymerase ß when either dATP, dGTP, or TTP was provided as the only nucleotide precursor. Since this preparation of recombinant polymerase β has been purified to homogeneity (21), the origin of this apparent degradation product is uncertain.

Non-templated addition by AMV reverse transcriptase and yeast DNA polymerase I

Figure 3 shows the results of an experiment in which AMV reverse transcriptase was incubated with the blunt-end duplex in the presence of all four dNTPs (Lane 2) or with individual dNTPs (Lanes

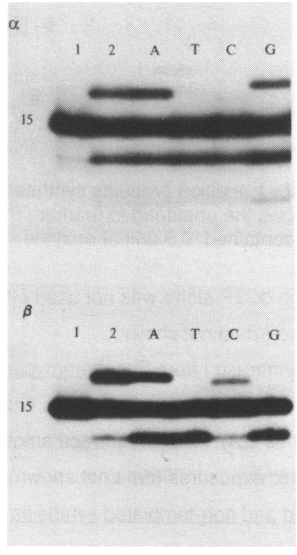

Figure 2. Electrophoretic analysis of +1 addition products synthesized by chick embryo polymerase α (top) and rat polymerase ß (bottom) on the duplex DNA substrate. In each case Lane 1 shows the unextended 15mer marker, Lane 2 shows the products synthesized in the presence of all four dNTPs, and the remaining lanes show the products synthesized in the presence of individual dNTPs. Polymerase α reactions contained 1.75 units of enzyme and polymerase ß reactions contained 1.8 units of enzyme.

A,T,C,G). This enzyme used dATP, and to a lesser extent dGTP, to carry out the +1 addition reaction when either dNTP was supplied individually; pyrimidine precursors were used much less efficiently. Interestingly, the presence of all four dNTPs in the reaction resulted in the addition of either dATP or dCTP with roughly equal efficiencies. This follows from the fact that the +1 product synthesized in the presence of all four dNTPS appears on the autoradiogram as a doublet (Figure 3, Lane 2). The upper and lower bands of the doublet, each of approximately equal intensity, have mobilities corresponding to the addition of dATP and dCTP, respectively. Therefore AMV reverse transcriptase used both dATP and dCTP to carry out blunt-end addition when all four dNTPs were

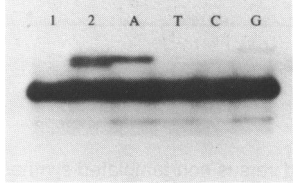

Figure 3. Blunt-end addition products synthesized by AMV reverse transcriptase. Samples are identical to those in Figure 2. Each reaction contained 1 unit of AMV reverse transcriptase.

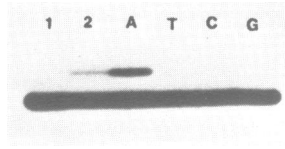

Figure 4. Electrophoretic analysis of +1 addition products synthesized by immunoaffinity-purified yeast DNA polymerase I. Lane 1 shows the unextended marker. The remaining samples are the same as those in Figure 2. Each reaction contained 0.5 unit of enzyme.

included in the reaction, even though dCTP alone was not used efficiently (Lane C). No addition to the single-stranded primer was observed (data not shown).

Immunoaffinity-purified DNA polymerase I from Saccharomyces cerevisiae also carried out the +1 addition reaction on the blunt-ended duplex (Figure 4). This enzyme preferentially used dATP to carry out the reaction (Figure 4, Lanes 2,A). Addition of trace amounts of dGTP and dCTP was also detected with longer autoradiographic exposures (data not shown).

Relative reaction rates for templated and non-templated synthesis

The preceding experiments do not address the important question of the rate at which non-templated nucleotide addition takes place as compared to normal template-directed synthesis. To obtain an estimate for the relative rates of these two processes, a 5' end-labeled, 14mer primer was annealed to an 18mer template to form the following substrate:

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5' 32P-CCTTTTCGTCGGCC      3'
3'     GGAAAAGCAGCCGGGTAG  5'.
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The rate of DNA synthesis on this substrate was determined by analyzing, on polyacrylamide gels, the products of the reaction as a function of time. (In this context the term rate refers to the overall rate of appearance of product. It therefore includes the effects of dissociation from and reassociation with the substrate in addition to possible differences in the catalytic rate constants for phosphodiester bond formation.) Addition of the first four nucleotides via templated synthesis

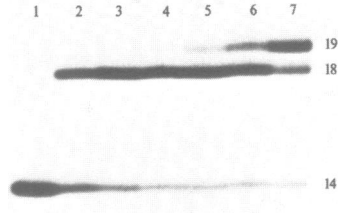

Figure 5. Relative rates of templated versus non-templated synthesis by AMV reverse transcriptase. Lane 1 shows the unextended 14mer primer. The remaining lanes show the reaction products synthesized in 30 sec (Lane 2), 1 min (Lane 3), 2 min (Lane 4), 5 min (Lane 5), 10 min (Lane 6), and 30 min (Lane 7). Product lengths are indicated alongside the figure. The reaction contained 8 units of AMV reverse transcriptase.

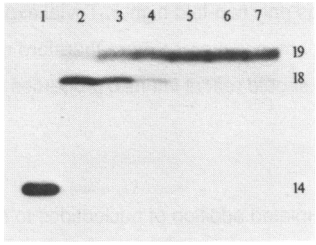

Figure 6. Relative rates of templated versus non-templated synthesis by yeast DNA polymerase I. Samples are identical to those in Figure 5. The reaction contained 28 units of enzyme. The enzyme preparation used in these experiments was not purified by immunoaffinity chromatography and is not homogeneous.

was monitored by the appearance of a full length, 18mer product on the autoradiogram. Non-templated addition was monitored by the appearance of a 19mer product. The results obtained with AMV reverse transcriptase are shown in Figure 5. The appearance of the 19mer band is significantly delayed relative to the appearance of the 18mer band. Quantitative analysis of this experiment is complicated by the requirement for some templated synthesis to occur in forming the blunt-end duplex substrate for non-templated addition. However, it is possible to obtain a rough estimate of the rate difference by noting that more than 50% of the starting primer has been extended to the end of the template within 30 seconds (Figure 5, Lane 2) whereas less than 50% of the 18mer has been converted to the 19mer in 10 minutes (Figure 5, Lane 6). If one assumes that the relative rate difference between templated and non-templated synthesis is reflected by the ratio of the times required to convert 50 % of the appropriate starting population to product, then the difference can be estimated as [(600 - 30)/30] x 4 = 76-fold faster for templated synthesis. In this calculation a correction was made for the time (<30 sec) required to form the substrate for non-templated addition. The factor of 4 was included to correct for the difference in the number of nucleotides incorporated during templated versus non-templated synthesis. This number represents a minimum estimate and the actual rate difference is likely to be several hundred-fold. A similar difference between the rates of templated and non-templated synthesis was obtained for DNA polymerase ß (data not shown).

The relative rate of non-templated addition for the α–type polymerases (chick embryo pol α and yeast pol I) was significantly faster than that observed for polymerase ß. The results for yeast polymerase I are shown in Figure 6. For this enzyme, the appearance of the 19mer (non-templated) product is clearly evident within 30 seconds (Figure 6, Lane 2), and roughly half of the 18mer has been converted to the 19mer within one minute (Figure 6, Lane 3). The rate of non-templated addition therefore appears to be within an order of magnitude of that for template-directed synthesis for yeast DNA polymerase I. Similar results were obtained for polymerase α from chick embryo (data not shown). Equivalent amounts of enzyme (as determined by assay on an activated DNA substrate) were used in the experiments with polymerase α and polymerase β; the amount of

yeast DNA polymerase I used was only two-fold higher. Trivial explanations for the difference seen between the α-type polymerases and polymerase β are therefore unlikely, and the difference in rates of non-templated synthesis should reflect intrinsic properties of these two classes of DNA polymerase.

DISCUSSION

The ability to catalyze non-templated addition of nucleotides to the 3' termini of blunt-ended DNA duplexes appears to be a general property of DNA polymerases since a number of different enzymes exhibit this phenomenon. These enzymes include DNA polymerases from several eucaryotes (chick embryo polymerase α, rat polymerase ß, and yeast DNA polymerase I), eubacteria [Taq DNA polymerase and DNA polymerase I (Klenow fragment) from E. coli; 14) and an avian retrovirus (AMV reverse transcriptase). All of the enzymes studied (with the possible exception of polymerase α; see Results) share a preference for the use of dATP to carry out blunt-end addition. This interpretation assumes that the differential electrophoretic mobility of the reaction products synthesized in the presence of individual dNTPs truly reflects the small differences in charge to mass ratio and/or secondary structure introduced into the oligonucleotides by the presence of different deoxynucleotides at their 3' termini. Direct sequence analysis of the +1 addition product synthesized by the Klenow fragment on a primer/template substrate revealed the expected DNA sequence and demonstrated that dAMP was preferentially added to the 3' terminus (14). Therefore we conclude that differential electrophoretic mobility accurately reflects the specificity of nucleotide addition. Since the "template" strand for these experiments does not contain any thymidine residues, the +1 addition of dATP cannot involve the use of coding information from the template. Therefore addition of dATP presumably occurs in a non-templated manner. An alternative possibility, that imperfect hybridization leaves a looped out base in the middle of the primer strand and an exposed 5' template base, is unlikely for the following reasons. First, a consideration of the sequence of the duplex reveals that such an event would eliminate the base pairing between the template and the 3' end of the primer strand, making the substrate unsuitable for a DNA polymerase. Moreover, even if such a structure could be used, the exposed 5' template base (guanine) would be expected to code for the (templated) addition of dCMP rather than the misinsertion of dAMP when all four dNTPs were provided. Since the preferred product represents the addition of dAMP, we conclude that the most probable mechanism is non-templated nucleotide addition. It is likely that the addition of nucleotides other than dATP also occurs in a non-templated fashion. However, we cannot exclude the formal possibility that some of these latter events, particularly the addition of dCMP by AMV reverse transcriptase, involve the use of coding information made available as a result of a transient misalignment of the primer/template substrate. It is of interest that many, if not all, DNA polymerases preferentially insert dATP opposite sites of base loss (non-coding apurinic or apyrimidinic sites) during DNA synthesis in vitro (22-25). The non-templated blunt-end addition reaction may be analogous to the insertion of nucleotides opposite abasic sites. We note, however, that an abasic site within a segment of DNA

does not present precisely the same structural features to a DNA polymerase as does a blunt-ended DNA duplex.

The rate at which non-templated addition takes place is generally slow compared to template-directed synthesis, although the magnitude of the difference depends upon the particular DNA polymerase studied. It should be emphasized that these experiments do not show that the catalytic rate constant for phosphodiester bond formation during non-templated synthesis is necessarily less than it is during templated synthesis. It is possible that the polymerase dissociates rapidly from the substrate after the completion of templated synthesis. According to this hypothesis, the rate-limiting step during non-templated synthesis would be the rate of reassociation of the polymerase with the (blunt-end) substrate. The α-type DNA polymerases (chick embryo pol α and yeast DNA polymerase I) appear to carry out non-templated addition at a significantly faster rate than polymerase β. In this regard it is worth noting that template-directed synthesis by polymerase β (in the presence of magnesium) is distributive whereas synthesis by α polymerases is semiprocessive (6). The faster rate of non-templated nucleotide addition by α-type polymerases may reflect this difference in processivity.

The ability to catalyze the addition of a nucleotide onto the 3' hydroxyl terminus of a DNA fragment without making use of coding information from a template strand is reminiscent of the activity of terminal deoxynucleotidyl transferase, an enzyme which adds nucleotides onto single-stranded DNA (12). However, the non-templated nucleotide addition reactions described in this report have a strict requirement for a duplex DNA substrate. It is possible that this "terminal transferase-like" activity common to both procaryotic and eucaryotic polymerases represents an evolutionary remnant of an enzymatic activity used by an ancestral enzyme prior to the advent of template-directed DNA synthesis. In this context it is noteworthy that rat polymerase ß and terminal transferase share significant amino acid sequence identity, suggesting that these polymerases were derived from a common ancestral gene (26,27).

The biological significance of the non-templated blunt-end addition reaction is not clear at present. However, given the low frequency with which spontaneous mutations occur in vivo (2-4), it is possible that some fraction of the mutations that arise as a consequence of replication errors could be due to non-templated nucleotide addition events occurring during DNA synthesis. In particular, non-templated additions might result in +1 frameshift mutations, with a bias towards the insertion of an A:T base pair as a result of preferential dAMP addition. For the eucaryotic enzymes, it is also possible that this capability could be exploited in vivo for processes such as telomere replication, perhaps in conjunction with accessory factors that modulate efficiency and/or specificity. In any event, the ability to carry out non-templated nucleotide addition demonstrates an interesting and unexpected property of DNA polymerases.

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