Primer-terminus Stabilization at the $\phi 29$ DNA Polymerase Active Site

MUTATIONAL ANALYSIS OF CONSERVED MOTIF TX$_2$GR

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The functional significance of the conserved motif TX$_2$GR, included in one of the six main regions of amino acid sequence similarity identified in the C-terminal portion of both *Escherichia coli* DNA polymerase I-like and eukaryotic-type DNA polymerases (Blanco, L., Bernad, A., Blasco, M.A., and Salas, M. (1991) *Gene* (Amst.) 100, 27–38) has been studied by site-directed mutagenesis in the $\phi 29$ DNA polymerase. A revised multiple alignment of this region, including 61 DNA polymerases belonging to these two superfamilies, is presented. In addition, based on amino acid sequence comparisons and by extrapolation to the crystal structure of T7 RNA polymerase, a similar motif (DX$_2$GR) is predicted to be structurally and functionally equivalent in RNA polymerases, the other class of DNA-dependent polymerases.

The severe defect in polymerization displayed by two of the $\phi 29$ DNA polymerase mutants used in this study (T434N and R438I) is interpreted as the consequence of a decreased capacity to stabilize the binding of primer-template DNA structures in a polymerization-competent conformation. These mutants were also severely affected in the formation of terminal protein (TP)-dAMP initiation complex, a reaction in which $\phi 29$ DNA polymerase is able to use the TP as primer.

Although DNA polymerases constitute a wide group of enzymes of prokaryotic and eukaryotic (viral or cellular) origin, several genetic and biochemical evidences led to the proposal that all DNA polymerases may have a similar structure. This claim has been supported in part by the study of the three-dimensional structures of the Klenow fragment of pol I (KF) and human immunodeficiency virus type I reverse transcriptase (reviewed by Joyce and Steitz (1994)). The proposed underlying similarity between all DNA polymerases has been also supported by sequence analysis and site-directed mutagenesis studies.

In addition to the generally conserved Klenow-like 3′–5′-exonuclease active site, located at the N-terminal portion of DNA polymerases (Bernad et al., 1989; Blanco et al., 1992a), several segments of amino acid similarity have been identified in the C-terminal portion of eukaryotic type DNA polymerases (Bernad et al., 1987; Larder et al., 1987; Wong et al., 1988; Blanco et al., 1991) and prokaryotic, pol I-like DNA polymerases (Ollis et al., 1985; López et al., 1989; Lawyer et al., 1989; Leavitt and Ito, 1989). Alternative alignments of these conserved C-terminal segments between the two families of DNA polymerases have been proposed (Matsumoto et al., 1988; Delarue et al., 1990; Blanco et al., 1991; Grabherr et al., 1992).

The role of particular residues belonging to the most conserved C-terminal motifs has been studied by site-directed mutagenesis in $\phi 29$ DNA polymerase (reviewed by Blanco and Salas (1994)) and other DNA-dependent DNA polymerases. Thus, motif YGDTS is probably involved in metal binding and catalysis in $\phi 29$ (Bernad et al., 1990), herpes (Dorsky and Crumpacker, 1990; Marcy et al., 1990), adenovirus (Joung et al., 1991), and human α (Copeland and Wang, 1993) DNA polymerases. Motif DX$_2$SLYP has been shown to be important for dNTP binding and/or catalysis in $\phi 29$ (Blasco et al., 1993a), T4 (Reha-Krantz et al., 1993), and human α (Dong et al., 1993a, 1993b) DNA polymerases. Residues of the motif KX$_2$NXXYG have been shown to be involved in primer-template binding and dNTP positioning in $\phi 29$ DNA polymerase (Blasco et al., 1992a, 1993b). Motif KXY has been proposed to contribute to primer binding in $\phi 29$ DNA polymerase (Blasco et al., 1992b). This proposal has been recently supported by analysis of highly purified mutant proteins in both Lys and Tyr residues.

Motif TX$_2$GR is present in both groups of prokaryotic type and eukaryotic type DNA polymerases (Blanco et al., 1991), except in the subgroup of TP-primed DNA polymerases, which contain a less strict motif, TX$_2$AR. Whereas the Arg residue belonging to the TX$_2$GR motif in KF has been shown to be important for DNA binding and critical for the catalysis of the reaction (Polesky et al., 1990, 1992), no site-directed mutagenesis studies in this motif of any eukaryotic type DNA polymerase have been described so far.

In this paper, we report the characterization of purified $\phi 29$ DNA polymerase mutant derivatives T434N, A437G, R438K, and R438I in motif TX$_2$AR. The results obtained indicate that mutants in residues Thr$^{63}$ and Arg$^{638}$ (i) have a decreased ability to bind a primer-template DNA structure; (ii) are partially affected in TP binding; and (iii) could be implicated, directly or indirectly, in catalysis. In support of the hypothesis that a similar active site could be conserved in all nucleic acid polymerases, we further report the identification of a similar motif (DX$_2$GR) in monomeric and multimeric DNA-dependent RNA polymerases.

**MATERIALS AND METHODS**

Nucleotides—[α-$^{32}$P]dATP (400 Ci/mmol) and [γ-$^{32}$P]ATP (5000 Ci/mmol) were obtained from Amersham International Plc. Unlabeled nu-
cleotides were from Pharmacia Biotech Inc.

**Proteins**—Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Wild-type 429 DNA polymerase was purified as described (Lázaro et al., 1994). 429 DNA polymerase site-directed mutants were purified essentially in the same form, as described (Zaballos et al., 1989).

**DNA Templates and Substrates**—TP-DNA was obtained as described (Péfalva and Salas, 1982). 429 DNA was obtained by proteinase K treatment of phage particles in the presence of SDS (Inciarte et al., 1987), followed by phenol extraction and ethanol precipitation. 429 DNA was digested with EcoRI to generate DNA fragments with 3'-overhangs (Polishko and Salas, 1982). 429 DNA was purified from the EcoRI-digested 429 DNA (1.6 pmol of DNA ends) as template, and the absence of any other changes were confirmed by complete sequencing of each 429 DNA polymerase mutant gene. 429 DNA polymerase under the control of the T7 RNA polymerase-specific T7 promoter (Tabor and Richardson, 1985). The presence of the desired mutations and the absence of any other changes were confirmed by complete sequencing of each 429 DNA polymerase mutant gene. Sequencing was carried out by the chain termination method (Sanger et al., 1977), using Sequenase version 2.0 from U.S. Biochemical Corp. and a set of synthetic oligonucleotide primers specific to 429 DNA polymerase as sequencing primers. Expression of the mutant proteins was carried out in E. coli strain BL21(DE3)LYsS (Studier, 1991).

**3'-5'-Exonuclease Assay**—The reaction mixture contained, in 12.5 µl, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 10 mM MgCl₂, 0.1 µM [α-32P]ATP (2.5 pCi), 0.1 µM each dGTP and d'TTP, 0.25 µg of EcoRI-digested 429 DNA (1.6 pmol of DNA ends) as template, and 10 ng of the corresponding DNA polymerase. After incubation for 15 min at 30 °C, the reactions were stopped and the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The Cerenkov radiation in the excluded volume was counted and used for quantitation. For qualitative analysis, the labeled DNA was denatured by treatment with 1 M NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels. After electrophoresis, the position of unit-length 429 DNA was detected by ethidium bromide staining, and then the gels were dried and autoradiographed.

**Interference Assay for TP Binding**—Reactions were carried out as described for the protein-primed initiation assay, in the absence of template and using a limiting amount of TP and different proportions of wild-type/mutant DNA polymerases. In the case of D249E, as a competition control, the amounts of proteins used were as follows: 12.5 ng of TP, 25 ng of wild-type DNA polymerase, and 25 or 100 ng of mutant D249E. For mutant T434AN, the amounts of proteins used were as follows: 12.5 ng of TP, 25 ng of wild-type DNA polymerase, and 12.5, 25, 50, or 100 ng of mutant T434AN. In both cases, the reactions were incubated at 30 °C for 3 h. In the case of mutants R438K and R438E, the amounts of proteins used were as follows: 2.5 ng of TP, 5 ng of wild-type DNA polymerase, and 5, 10, 15, or 20 ng of mutant proteins. Incubation was for 15 h at 30 °C. In all cases, after incubation, the samples were stopped and analyzed as described for the initiation reaction.

**TP-DNA Replication (Protein-primed Initiation Plus Elongation)**—The reaction mixture contained, in 25 µl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.25 µg of TP-DNA, 25 ng of purified TP, and 10 ng of the corresponding DNA polymerase. After incubation for the indicated times at 30 °C, the reactions were stopped and the samples were filtered as described above. The Cerenkov radiation in the excluded volume was counted and used for quantitation. For qualitative analysis, the labeled DNA was denatured by treatment with 1 M NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels. After electrophoresis, the position of unit-length 429 DNA was detected by ethidium bromide staining, and then the gels were dried and autoradiographed.

**RESULTS**

The **TX. GR Motif of DNA-dependent DNA Polymerases**—The consensus sequence TX. GR is included in one of the most significant homology regions present in both superfamilies of pol 1-like and eukaryotic type DNA polymerases (Blanco et al., 1991). Fig. 1 shows an updated multiple alignment of this region, in which 61 sequences of DNA polymerases belonging to...
FIG. 1. The TX,GR motif of DNA-dependent DNA polymerases. The multiple alignment of the amino acid sequences containing motif TX,GR would be located in the turn between β-strands 7 and 8. DNA polymerase nomenclature and sequence references are compiled in Braithwaite and Ito (1993), with the exception of the subgroup of protein-priming DNA polymerases (indicated as relative to the N terminus of each DNA polymerase.

According to the three-dimensional structure of the KF (Ollis et al., 1985), motif TX,GR would be located on the turn between β-strands 7 and 8, being part of the polymerization active site. Furthermore, as indicated in the alignment shown in Fig. 1, the residues preceding those forming β-strand 7 in pol I also show traces of similarity with those preceding β-strand 8 in pol I (involving motif TX,GR). This apparent structural duplication can also be observed in other DNA polymerase sequences aligned in Fig. 1. In the case of protein-primed DNA polymerases, a specific insertion preceding the region aligned with pol I β-strand 7 has been previously reported (Blanco et al., 1991; see also Fig. 1).

Construction of Site-directed Mutants in the TX,GR Motif—To analyze the effect of mutations in each of the three most conserved amino acid residues of this region in φ29 DNA polymerase (Thr434, Ala437, and Arg438), single changes were designed taking into account secondary structure predictions (Chou and Fasman, 1978; Garnier et al., 1978) and general suggestions for conservative substitutions (Bordo and Argos, 1991). Mutants T434N, A437G, R438K, and R438I were obtained. The change of Thr434 into Asn was designed to remove the hydroxyl group of the side chain. The change of Ala437 into Gly restored the consensus motif TX,GR, present in most DNA polymerases that use proteins as primers, where this Gly residue is always substituted by Ala, Ser, or Thr. Interestingly, DNA polymerases Chlorella viruses NY-2A and PBCV-1 have the sequence VTATGR, identical to that in pol I (see Fig. 1). To estimate the significance of the alignment shown in Fig. 1, we used the sequence VTAGR to search protein data bases. Out of 37 Brattleboro proteins searched, this sequence was only found in four proteins: the three polymerases mentioned above and an ATP synthase from Anabaena sp.

3′-5′-Exonuclease Activity of φ29 DNA Polymerase Mutants—In agreement with the proposed location of the 3′–5′-exonuclease active site of φ29 DNA polymerase in the N-terminal portion (residues 1–190) of the protein (Bernard et al., 1989; Blanco et al., 1991), the four mutant derivatives in con-
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FIG. 2. DNA polymerase/exonuclease coupled assay. The assays were carried out as described under “Materials and Methods,” using 32P-labeled hybrid molecule sp1/sp1c+6 as primer/template DNA and the indicated concentration of each dNTP. The DNA polymerase used in each case is indicated. Arrows indicate the 15-mer position (nonelongated primer) and 21-mer position (completely elongated primer).

served residues Thr434, Ala437, and Arg438 had essentially the wild-type level of 3'-5'-exonuclease activity, measured on single-stranded DNA as substrate (not shown).

DNA-primed Polymerization Activity of Mutant φ29 DNA Polymerases—The effect of the mutations on the ability of the enzyme to catalyze DNA polymerization onto a DNA primer molecule was first analyzed in a short polymerization assay (filling in of φ29 DNA-EcoRI ends) as described under “Materials and Methods.” In this assay, the enzyme catalyzes the incorporation of few nucleotides onto a DNA primer, and polymerization properties such as strand displacement and processivity are not required. On the other hand, the analysis of the 3'-5'-exonucleolytic release of dAMP coupled to this filling in reaction (turnover) allows us to estimate the stability of incorporated nucleotides. As shown in Table I, mutants T434N and R438I were almost inactive in DNA polymerization, whereas mutants A437G and R438K showed 80 and 70%, respectively, of the activity of the wild-type DNA polymerase. The analysis of the dAMP release during this short polymerization assay revealed that proteins T434N and R438I have about 5- and 7-fold higher turnover, respectively, than that of the wild-type enzyme. This phenotype is generally interpreted as the consequence of either an inefficient translocation or a defective stabilization of the primer terminus.

Using a 5'-labeled oligonucleotide (15-mer) hybridized to a larger template oligonucleotide (21-mer), it is possible to analyze the equilibrium between exonuclease and polymerization, since the products of the two activities on this primer-template structure can be simultaneously detected. By addition of increasing amounts of dNTPs, this equilibrium is displaced toward synthesis, exonuclease being competed by DNA polymerization. Whereas mutant polymerases R438K (Fig. 2, panel C) and A437G (not shown) had the same dNTP requirement for polymerization as the wild-type DNA polymerase (panel A), mutant proteins T434N (panel B) and R438I (panel D) needed a 20-fold greater dNTP concentration than the wild-type enzyme for full polymerization.

The processivity and strand displacement capacity of the mutant derivatives during DNA polymerization were analyzed in a primed M13 DNA replication assay. As shown in Table I, mutant A437G had an activity slightly higher than that of the wild-type enzyme, and mutant R438K was partially active. Size analysis of the reaction products by electrophoresis in alkaline gels revealed that these mutations are not affecting processivity and strand displacement (results not shown). On the contrary, mutant proteins T434N and R438I were almost inactive (see Table I) in spite of the fact that the dNTP concentration used was 5-fold higher than that required to complete replication in a pol/exo coupled assay.

Gel Retardation of Primer-template DNA Molecules by Wildtype and Mutant DNA Polymerases—The binding of the different mutant derivatives of the φ29 DNA polymerase to a primer-template DNA (sp1/sp1c+6) was analyzed by gel retardation assays, as described under “Materials and Methods.” Mg2+ ions strongly stimulate the formation of an enzyme-DNA complex that is competent for DNA polymerization, giving rise to a
single retardation band whose intensity depends on the amount of DNA polymerase used. As shown in Fig. 3, DNA retardation by mutant A437G was essentially similar to that of the wild type; mutant T434N retarded a lower amount of DNA; and mutant R438I only produced a faint retardation band. However, when Arg residue was changed into Lys, the DNA binding activity was only slightly affected. These results strongly suggest that both Thr and the positively charged Arg are important for primer-template binding at the polymerization active site.

Protein-primed TP-DNA Replication with the Mutant DNA Polymerases—Replication of TP-DNA starts at both DNA ends by a specific protein-priming mechanism and requires high processivity and strand displacement activity during elongation. Based on the fact that these properties are intrinsic to φ29 DNA polymerase, efficient in vitro synthesis of full-length φ29 DNA (19,285 base pairs) can be achieved in the presence of one or more divalent metal activators, and translocation of the enzyme on the template track prior to the next cycle of polymerization. In some polymerases, catalysis is both preceded and followed by nonchemical steps, frequently described as conformational changes in the enzyme (Patel et al., 1991; Dahlberg and Benkovic, 1991). Furthermore, DNA polymerases that support protein priming (exemplified by bacteriophage φ29 DNA polymerase) must also interact with a free molecule of the TP that is used as primer to initiate replication.

From the examination of the three-dimensional structures of the KF, human immunodeficiency virus reverse transcriptase, and T7 RNA polymerase and from genetic and biochemical studies, it has been proposed that all nucleic acid polymerases may be variations of a common structure (reviewed by Joyce and Steitz (1994)). Analysis of protein sequences has provided structure-function correlations from site-directed mutagenesis studies, it is important to bear in mind that a single substitut-
Role of Motif TX$_2$GR in DNA-dependent DNA Polymerases—By analysis of the three-dimensional structure of the KF, the polymerization domain has been divided into three subdomains, named palm, fingers, and thumb. The palm subdomain, the most conserved part among the different DNA polymerases, contains 3 carboxylate residues Asp$_{59}$, Asp$_{22}$, and Glu$_{46}$ that may serve to anchor a pair of divalent metal ions (reviewed by Joyce and Steitz (1994)). Polymerization catalysis is proposed to occur by a two-metal ion mechanism, analogous to that described for the 3′-5′-exonuclease reaction (Beese and Steitz, 1991). Besides, a number of other residues (e.g. Arg$_{40}$, Lys$_{70}$, and Gin$_{43}$) have been shown to be important in accelerating the reaction. One of these residues, Arg$_{40}$, belongs to the motif TX$_2$GR, which forms part of the palm subdomain. Substitution of Arg$_{40}$ into Ala produced one of the most deleterious mutations isolated in KF; its affinity for DNA was 20-fold lower than that of the wild-type enzyme, and its $k_{cat}$ was diminished about 400-fold (Poleski et al., 1992).

By site-directed mutagenesis, we have demonstrated that the related motif TX$_2$AR of φ29 DNA polymerase is important for DNA binding and also for interaction with the primer TP. Summarizing, single substitution of residues Thr$_{434}$ and Arg$_{438}$ resulted in (i) an inefficient binding to primer-template structures at the polymerization active site, which could be responsible in a conserved residue of a DNA polymerase may alter one (or several) of the individual steps mentioned above, therefore affecting the overall process of DNA synthesis.

**Fig. 3.** Gel retardation of primer-template DNA molecules by wild-type or mutant DNA polymerases. A labeled hybrid molecule of two oligonucleotides (21/15-mer) was incubated either with the wild type or with the indicated mutant DNA polymerase, in the conditions described under "Materials and Methods." The bands corresponding to free 21/15-mer DNA and to the DNA polymerase-DNA complex are marked with arrows. The amount of DNA polymerase is indicated in each case.

**Fig. 4.** TP-DNA replication. The reactions were carried out as indicated in "Materials and Methods" in the presence of 25 ng of TP and 10 ng of the indicated DNA polymerase. The time of incubation is indicated. After quenching, samples were electrophoretically analyzed in alkaline-agarose gels. The mobility corresponding to unit-length TP-DNA (19,285 nucleotides) is indicated.

**Fig. 5.** A, template-dependent or template-independent formation of TP-dAMP complex catalyzed by the wild-type or mutant DNA polymerases. The reactions were carried out in the conditions indicated under "Materials and Methods," in the presence of 25 ng of TP and 10 ng of the corresponding DNA polymerase. The template-dependent reaction was carried out in the presence of 10 mM MgCl$_2$ and 0.5 μg of TP-DNA. Incubation was for 5 min at 30 °C. The template-independent reaction was carried out in the presence of 1 mM MnCl$_2$. Incubation was for 14 h at 30 °C. B, Interference assay for TP binding. The assay of template-independent formation of TP-dAMP complex by the wild-type DNA polymerase was performed in the presence of the mutant DNA polymerases T434N, R438K, or R438I, which cannot carry out this reaction (see part A). An inactive DNA polymerase whose interaction with the TP was normal would inhibit the formation of the TP-dAMP complex by competition for the TP with the wild-type DNA polymerase. The previously characterized inactive mutant D249E (Blasco et al., 1993a) was used as a control of 100% competition, since its inhibition profile was identical to the theoretical one. The TP-dAMP formed in the different competition conditions relative to that formed in the absence of competition (100%) is indicated.
A

DNA-dependent polymerases

\begin{itemize}
  \item Pol I-like DNA polymerases
  \item Eukaryotic-like DNA polymerases
  \item Monomeric RNA polymerases
  \item Multimeric RNA polymerases (largest subunit)
\end{itemize}

B

DNA-dependent polymerases

\begin{itemize}
  \item Pol I-like DNA polymerases (E. coli Pol I)
  \item Eukaryotic-like DNA polymerases (p29 DNA pol)
  \item Monomeric RNA polymerases (T7 RNA pol)
  \item Multimeric RNA polymerases (E. coli \( \beta' \) subunit)
\end{itemize}

**FIG. 6.** Conserved amino acid regions in different families of DNA-dependent polymerases. A, pol I and p29 DNA polymerases were selected as representatives of the pol I-like and eukaryotic type superfamilies of DNA-dependent DNA polymerases, respectively. Relevant amino acid similarity among the different classes of polymerases is indicated in white letters over a black background. Residues belonging to the motif \( TX_{GR} \) or \( DX_{GR} \) are marked with asterisks. The group of monomeric RNA polymerases includes proteins from bacteriophages T7 and SP6, linear plasmids Kalilo, pClK1, S-2, and pEM, and the mitochondrial enzyme from \( S. \) cerevisiae (S.C. RP041). All RNA polymerase sequences are reviewed in Chang et al. (1991) except that of T7 (Moffatt et al., 1984). Among the group of multimeric RNA polymerases (largest subunit), sequence references for \( E. \) coli (Ex.), the cyanobacterium \( N. \) commune (N.c.), liverwort (M.p.), tobacco (N.t.), spinach (S.O.), rice (O.s.), and maize chloroplasts (Z.m.) are reviewed in Igloi et al. (1990). Sequence references for RNA polymerase I from \( P. \) falciparum (P.f.) and mouse (M.m.) Drosophila melanogaster (D.m.) and \( T. \) brucei (T.b.) can be found in Li et al. (1989). Sequences from RNA polymerases II and III from \( S. \) cerevisiae can be found in Allison et al. (1985). Sequences from RNA polymerases from African swine fever virus (ASFV) and vaccinia were taken from Yáñez et al. (1993). B, the enzymes (or subunit) selected as representative of each class of DNA-dependent polymerases are indicated in parenthesis. Each selected sequence is represented by a straight line, with the N terminus at the left. In \( E. \) coli pol I and p29 DNA polymerase, I, II, and III indicate the three highly conserved motifs \( \text{exo} \) I, \( \text{exo} \) II, and \( \text{exo} \) III, proposed to form an evolutionary conserved 3'-5'-exonuclease active site (Bernad et al., 1989). In the \( E. \) coli pol I sequence, the N-terminal portion outside the 3'-5'-exonuclease domain contains another degradative activity, a 5'-3'-exonuclease. According to Blanco et al. (1991), in \( E. \) coli pol I and p29 DNA polymerase, regions 1-5 correspond to regions of amino acid similarity among the two superfamilies of DNA-dependent DNA polymerases. Specific insertions (SI 1 and SI 2) in p29 DNA polymerase were previously described (Blanco et al., 1991). Regions I to VII in T7 RNA polymerase correspond to the highly conserved regions of amino acid similarity among monomeric RNA polymerases, as described by Masters et al. (1987). A, B, and C stand for the three amino acid sequence motifs proposed to be conserved among DNA-dependent polymerases (Delarue et al., 1990). Regions I to VII in the largest subunit of multimeric RNA polymerases correspond to the conserved regions of amino acid similarity described by Allison et al. (1985). The portion aligned in part A, indicated by a shadowed bar, was used as an alignment axis.
sible of the severe loss of DNA primer-dependent synthetic activities; (ii) a defective interaction with the primer TP, that impairs TP primer-dependent synthetic reactions, both in the presence and absence of DNA template; (iii) a strong exonucleolytic activity on primer-template molecules, as determined by the analysis of the turnover coupled to DNA polymerization.

All of these results suggest a direct role of the Thr and Arg residues of this motif in the stabilization of the primer molecule (DNA or TP) at the polymerase active site. On the other hand, the substitution A437G, which converts the specific motif TX,GR to TX,RR, supports the existence, in addition to motifs A, B, and C, of a more extensive structural similarity between these enzymes. Moreover, the mutational analysis reported here for d29 DNA polymerase could be extrapolated to include other DNA-dependent DNA polymerases containing a consensus TX,GR motif.

Is There a Similar Conserved Motif in DNA-dependent RNA Polymerases?—By amino acid sequence comparisons (Delarue et al., 1990), DNA-dependent DNA polymerases and the monomeric class of DNA-dependent RNA polymerases were proposed to be structurally and perhaps functionally related by the presence of three amino acid sequence motifs, designated A, B, and C (see Fig. 6A). This proposal was further supported by mutational analysis (Polesky et al., 1990, 1992; Bonner et al., 1992; Osumi-Davis et al., 1992) and by comparison of the x-ray structures of the two most representative enzymes belonging to both groups: KF (Ollis et al., 1985) and T7 RNA polymerase (Sousa et al., 1993). In both cases, single mutations at highly conserved residues forming motifs A, B, and C were critical for the synthetic functions of these enzymes. Moreover, both crystal structures show that motifs A, B, and C adopt a nearly identical three-dimensional architecture, building both DNA and RNA polymerization active sites (Sousa et al., 1993).

Taking into account these structure-function relationships and the functional importance of residues belonging to the motifs TX,GR in DNA-dependent DNA polymerases (Polesky et al., 1990, 1992; this paper), we looked for a similar motif in the group of monomeric DNA-dependent RNA polymerases. As shown in Fig. 6A, an amino acid segment of about 27 residues in monomeric RNA polymerases could be aligned with region 2b (containing the TX-GR motif) of DNA polymerases shown in Fig. 1, exemplified here by pol I and d29 DNA polymerases. According to this alignment, a similar motif, DX,GR, included in one of the most conserved regions (region III, according to Masters et al. (1987)) aligns with motif TX,GR of DNA polymerases.

A detailed comparison of the structures of KF and T7 RNA polymerase supports the existence, in addition to motifs A, B, and C, of a more extensive structural similarity between these two enzymes (Sousa et al., 1993). However, it has not been emphasized that β-strands 7 and 8 of KF (forming part of the palm subdomain and containing motif TX,GR) have identical counterparts (β-strands 3 and 4) in T7 RNA polymerase. Taking into account the alignment shown in Fig. 6A and the results of Sousa et al. (1993), we predict that the DX,GR motif could be located in the turn between β-strands 3 and 4 of T7 RNA polymerase.

Allison et al. (1985) described the existence of two segments of amino acid similarity between the largest subunits of E. coli RNA polymerase (β) and those of RNA polymerases II and III of Saccharomyces cerevisiae, with the DNA-dependent DNA polymerase I from E. coli. One of these segments (region II in Fig. 6A) contains motif DX,GR (Fig. 6A) and is one of the most conserved among the largest subunits of multimeric RNA polymerases; no similar motifs were found in other subunits. The fact that motif DX,GR is restricted to an RNA polymerase subunit thought to be mainly involved in DNA binding is in agreement with the hypothesis that this motif could be involved in the DNA primer-template binding at a general polymerization active site.

The proposed alignment of this region constitutes the first report of amino acid sequence similarity among monomeric and multimeric DNA-dependent RNA polymerases. It is worth noting that motif B, located at the fingers subdomain, is not present in RNA-dependent DNA polymerases. This absence suggested that the fingers region could be related with the ability to bind and/or recognize either DNA or RNA as template (Delarue et al., 1990). Similarly, neither motif TX,GR nor motif DX,GR could be found in RNA-directed polymerases. Therefore, it is tempting to speculate that these motifs are directly involved in binding and stabilization of primer-template structural motifs and, consequently, playing an indirect but critical role in catalysis.

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