Volume 228, number 1, 144–148 FEB 05551 February 1988

The influence of a double-stranded hindrance on DNA synthesis performed by DNA polymerase α, T_4 DNA polymerase, DNA polymerase I (Klenow fragment) and AMV reverse transcriptase

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The influence of a double-stranded region on DNA synthesis performed by a series of DNA polymerases on a single-stranded template was studied. Two types of double-stranded hindrances were employed: a stable hairpin formed by the template alone and a region formed by the template and an extraneous oligonucleotide complementary to the template. While T_4 and calf thymus α DNA polymerases are strongly arrested at the beginning of either of the two double-stranded hindrances, the Klenow fragment of *E. coli* DNA polymerase I and avian myeloblastosis virus reverse transcriptase can pass through the double-stranded regions. The DNA chain-elongation rate seems to be undisturbed in the case of reverse transcriptase but greatly reduced for DNA polymerase I.

Hairpin structure; Double-stranded hindrance; Arrest point; DNA polymerase α; DNA polymerase T_4; DNA polymerase I; Reverse transcriptase

1. INTRODUCTION

The investigation of DNA synthesis performed by different DNA polymerases on a single-stranded template [1–6] indicates that there are two classes of DNA synthesis arrest sites: (i) palindromic sequences which form a hairpin structure; (ii) ordinary sequences which cannot form a secondary structure of this type. The pausing within these regions is a sequence-dependent rather than a spatial structure dependent event. Different DNA polymerases appear to have differing degrees of sensitivity to both factors. The pause positions for various DNA polymerases were found to be entirely different for the same DNA region used as template. Moreover, addition of two enzymes to the same incubation mixture resolved the pauses for each origin of polymerase. These results cannot be ascribed solely to the secondary structure of the template.

We constructed a special system to monitor the DNA synthesis elongation reaction on the same DNA sequence in the presence of two alternative double-stranded hindrances to the elongation reaction. Fig.1 depicts the organization of the system: either (i) part of a single-stranded template forms a stable hairpin or (ii) oligonucleotide R6, complementary to part of this palindromic sequence, being annealed to the template prevents formation of the hairpin structure, but instead gives rise to a double-stranded hindrance with the 5′-border located 10 nucleotides downstream of that of the hairpin. Comparison of DNA synthesized on the templates enables us to determine the precise locations of the arrest points resulting from both these kinds of secondary structures.

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2. MATERIALS AND METHODS

2.1. *Enzymes*

The Klenow fragment of DNA polymerase I (*E. coli*), DNA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

polymerase α (6 S, calf thymus), AMV reverse transcriptase (avian myeloblastosis virus) and T_4 polynucleotide kinase were from our laboratory stocks. Bacteriophage T_4 DNA polymerase was donated by Dr U.K. Koslov (Institute of Molecular Biology, Moscow).

2.2. *Oligonucleotides and DNA*

The oligonucleotide A1, CCCAGTCACGACGT, was kindly supplied by Dr A. Azhayev (Institute of Molecular Biology, Moscow). The oligonucleotide R6, AATTGTTATCCGCTCACAATTCC, was kindly provided by Dr B. Muller-Hill (Cologne University, FRG). DNA from bacteriophage M13 mp10 [7] was prepared as in [8].

2.3. *Oligonucleotide-template complexes*

Oligonucleotide A1 was ^{32}P-labelled at the 5'-termini by T_4 polynucleotide kinase as described [9]. Two types of primer-template mixtures were prepared (fig.1). The first contained 1.2 pmol single-stranded M13mp10 DNA and 1 pmol labelled oligonucleotide A1 in 30 μl of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA. The other comprised additionally 5 pmol unlabelled oligonucleotide R6. Mixtures were incubated at 70°C for 15 min and cooled to room temperature for several hours.

2.4. *DNA synthesis*

Reaction conditions were as follows:

T_4 DNA polymerase: 20 mM Tris-HCl (pH 8.4), 10 mM $MgCl_2$, 1.6 mM 2-mercaptoethanol, 50 mM NaCl, 10 μM of each dNTP, 6 μl primer-template mixture (0.24 pmol DNA), 3 U T_4 DNA polymerase; total volume, 30 μl; incubation temperature, 22°C.

DNA polymerase α: 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1.6 mM 2-mercaptoethanol, 10 μg/ml BSA, 10 μM of each dNTP, 6 μl primer-template mixture, 1 U enzyme; total volume, 30 μl; temperature, 37°C.

Reverse transcriptase and Klenow enzyme: 20 mM Tris-HCl (pH 8.4), 10 mM $MgCl_2$, 50 mM NaCl, 1.6 mM 2-mercaptoethanol, 1 μM of each dNTP, 6 μl primer-template mixture, 6 U reverse transcriptase or 0.2 U DNA polymerase I; temperature, 22–37°C for DNA polymerase I or 37–42°C for reverse transcriptase; volume, 30 μl.

Reactions were initiated by enzyme addition; 3-μl aliquots were withdrawn at the indicated times and mixed with 3 μl of 20 mM EDTA to terminate the reaction. The products were analyzed on 8% polyacrylamide gel electrophoresis under denaturing conditions.

DNA sequencing was accomplished using the chain-termination method of Sanger et al. [10], except that 3'-fluoro-2',3'-dideoxyribonucleoside 5'-triphosphates (3'-F-dNTP) were used for chain termination [11].

3. RESULTS AND DISCUSSION

3.1. *DNA polymerases α and T_4*

DNA polymerases α and T_4 were arrested at the 5'-end of a double-stranded hindrance in accor-

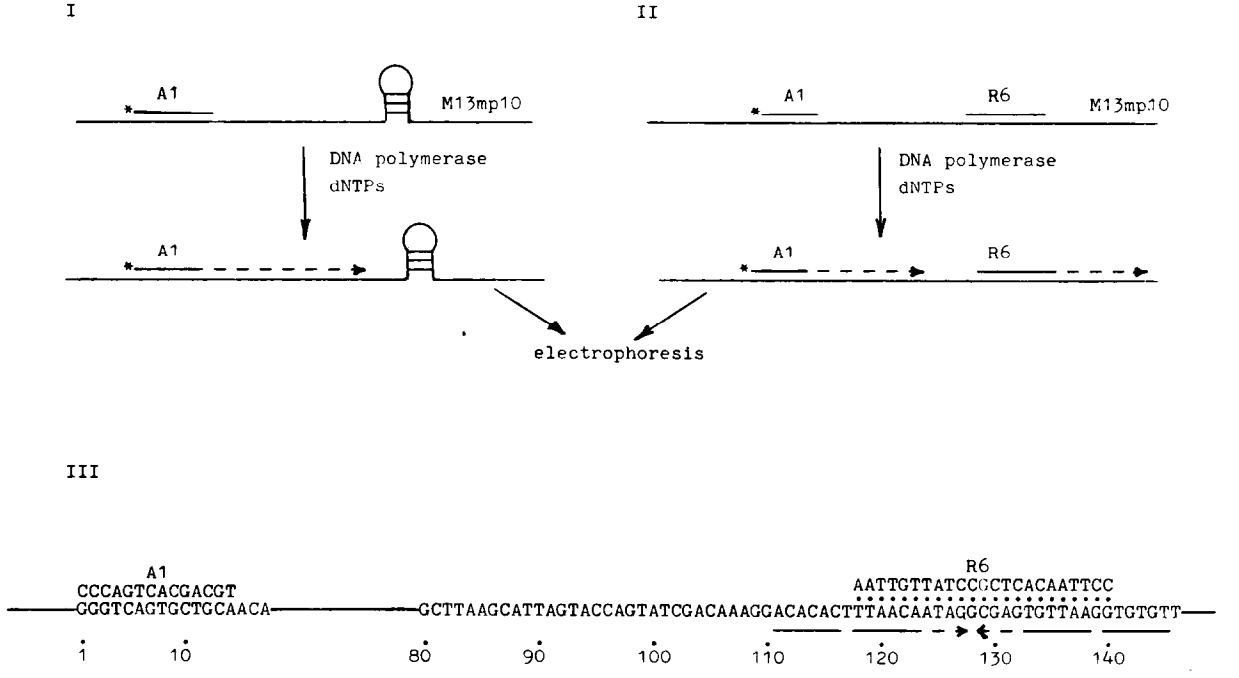

Fig.1. Scheme illustrating the experiment protocol. I, DNA synthesis in the presence of hairpin structure; II, synthesis in the presences of oligonucleotide R6; III, part of the DNA sequence of M13mp10 near the positions of oligonucleotides A1 and R6. The palindromic sequence is underlined. Numbering of nucleotides commences from the 5'-end of oligonucleotide A1.

dance with the observations of Weavwer and De Pamphilis [4] and Reckman et al. [6]. The major site of arrest observed in the absence of oligonucleotide R6 (fig.1,I) is located at nucleotides 109 and 110, both of which are C (fig.2). In the presence of oligonucleotide R6 (fig.2,II) there are no strong bands near the 109th C, but instead accumulation of DNA chains in the vicinity of the 117th A, the latter preceding the double-stranded hindrance, is clearly observed. Some products penetrate into the double-stranded part of the template for 2–3 nucleotides, but the intensities of the bands gradually diminish.

T_4 DNA polymerase behaves very much like DNA polymerase α in either the absence or presence of oligonucleotide R6. The pauses are merely shifted one nucleotide downstream when compared to that of DNA polymerase α (fig.2).

3.2. *Reverse transcriptase*

Comparison of the products of DNA synthesis performed by reverse transcriptase in the presence

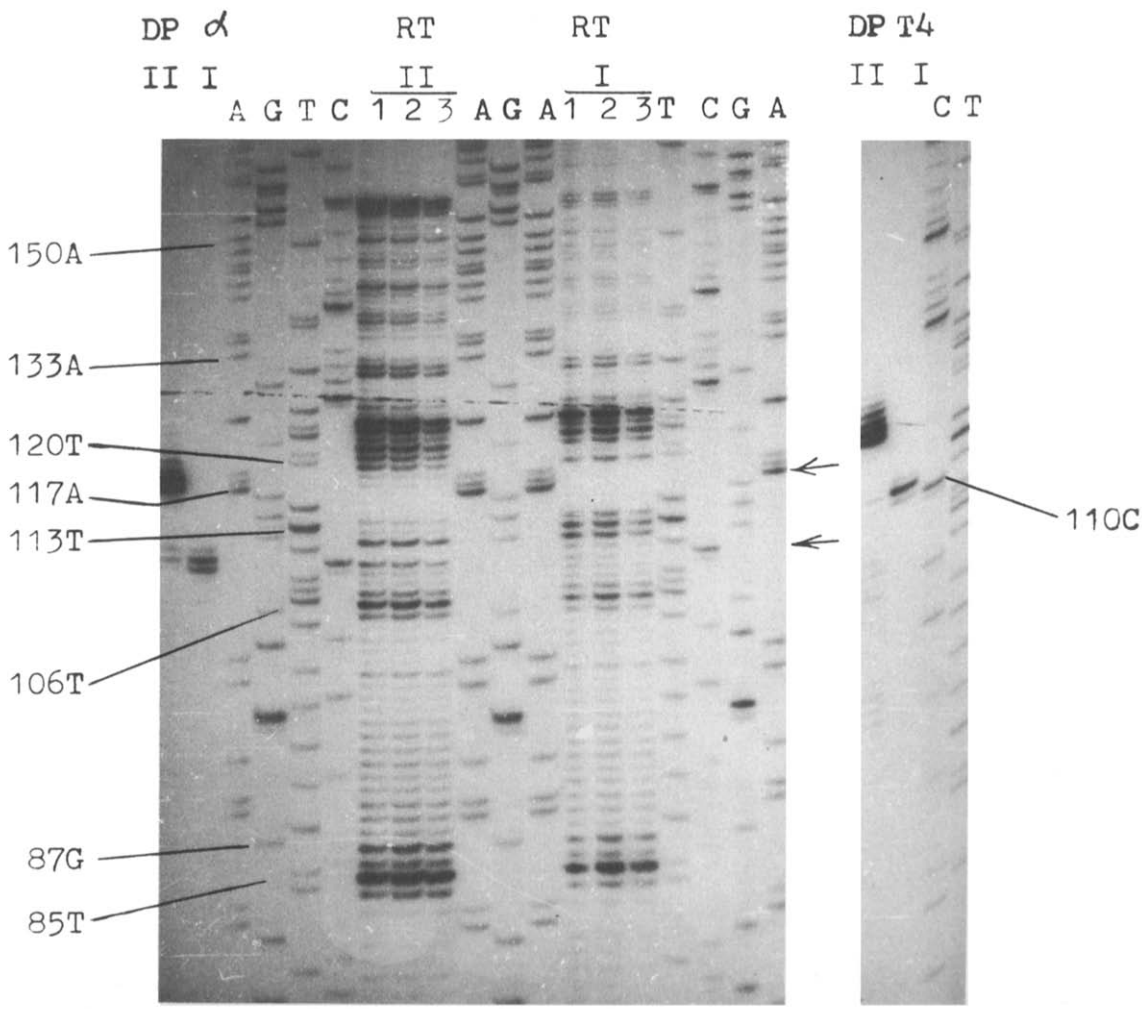

Fig.2. DNA synthesized by reverse transcriptase and DNA polymerases α and T_4 in the absence (I) or presence (II) of oligonucleotide R6. Single-stranded DNA of bacteriophage M13mp10 was used as template and ^{32}P-oligonucleotide A1 as primer. Reaction times: reverse transcriptase – (1) 15 min, (2) 30 min, (3) 60 min; DNA polymerase T_4 – 30 min (DP T4); DNA polymerase α – 30 min (DP α). DNA sequence reactions were performed according to Sanger's procedure with DNA polymerase I (Klenow fragment) in the presence of: A, dATP(3′-F); G, dGTF(3′-F); C, dCTP(3′-F); T, dTTP(3′-F). The final nucleotide preceding the hairpin structure and oligonucleotide R6 are indicated by arrows.

and absence of oligonucleotide R6 (fig.5,I and II) reveals only a minor influence of the secondary structure of the DNA on the pause distribution (cf. intensities of the 85th and 106th bands with those of the bands above the 109th C, especially the 110th, 113th and 120th nucleotides; fig.2,I and II). However, there is still no strong arrest point in either the presence or absence of oligonucleotide R6 near the beginning of the double-stranded hindrance. The presence of very long product chains indicates that reverse transcriptase could pass through the double-stranded part of the DNA template, obviously by displacement of the second strand.

3.3. *DNA polymerase I (Klenow fragment)*

DNA chain elongation by DNA polymerase I (Klenow fragment) is not arrested by hairpin structures (fig.3): DNA chains of more than 200 nucleotides in length are clearly visible on autoradiograms. Moreover, there is no strong pause at the beginning of the palindromic sequence (fig.3,I, positions 109–110). A moderate pause is located at positions 112–114, occurring four nucleotides downstream of the beginning of palindromic sequence (fig.1). Attribution of the pause to the presence of a hairpin structure is confirmed by the fact that the 112–114th bands are absent from the DNA products synthesized in the

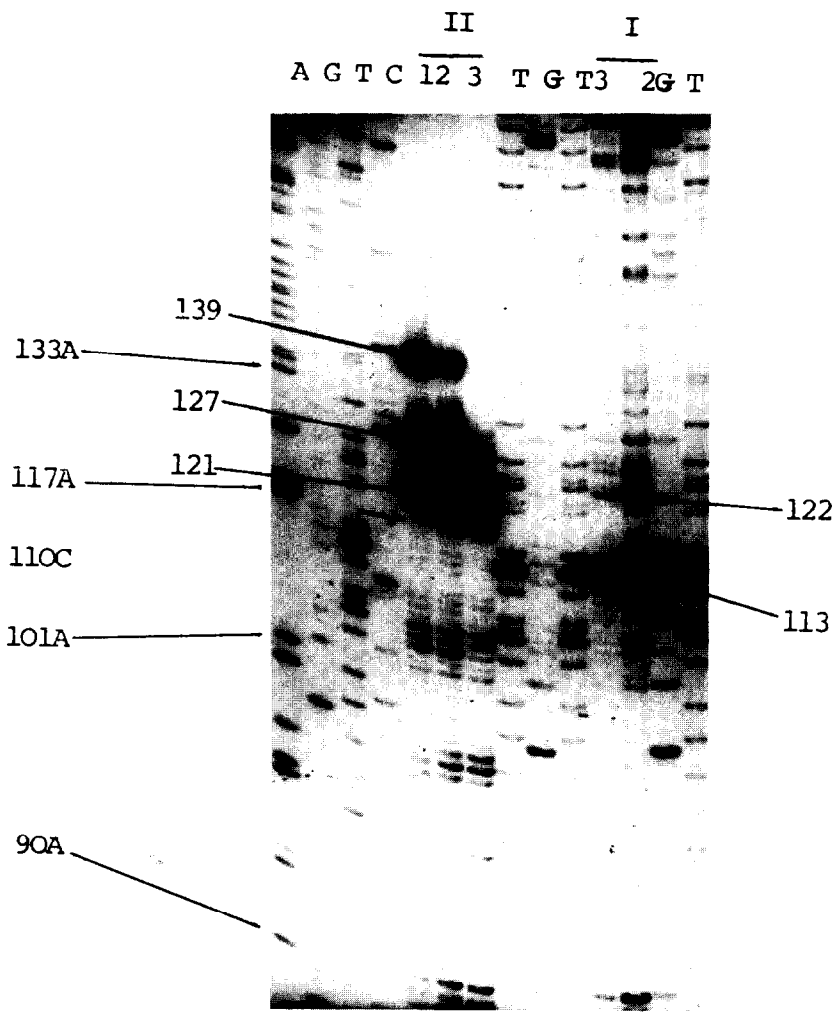

Fig.3. DNA produced by DNA polymerase I (Klenow fragment) in the absence (I) or presence (II) of oligonucleotide R6. Reaction time: (1) 4 min, (2) 16 min, (3) 60 min. Other symbols: see fig.2.

presence of oligonucleotide R6. Instead, the polymerase is arrested at nucleotide 121 of the nascent chain (fig.3,II) which is the 4th nucleotide downstream of the 5′-end of R6. There are also other strong pauses (near the 127th and 131th nucleotides) located far within the double-stranded region. However, synthesis does not proceed very far into this region (fig.3,II), indicating a low chain-elongation rate within the double-stranded region for the enzyme.

4. CONCLUSIONS

T_4 DNA polymerase and calf thymus DNA polymerase α are fully arrested by any double-stranded hindrances. The Klenow fragment of *E. coli* DNA polymerase I and AMV reverse transcriptase are able to pass through the double-stranded regions. The rate of DNA chain elongation appears to remain unaltered for reverse transcriptase but is greatly reduced for DNA polymerase I on proceeding through the double-stranded regions. The result does not appear to be sensitive to variations in temperature (see section 2 for limits), salt, pH or dNTP concentration whereas variation of these parameters does not prevent DNA synthesis proceeding at any DNA region. The present data indicate that the reverse transcriptase might be useful for the 'PCR amplification' procedure [12] instead of the Klenow fragment of DNA polymerase I.

Acknowledgements: We are grateful to our colleagues Drs Z.G. Chidgeavadze, L.P. Savochkina, T.V. Svirjaeva, A.Ya. Shevelev and A.A. Chenchik for enzymes and helpful discussions.

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