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Improved band shift assay for the simultaneous analysis of protein–DNA interactions and enzymatic functions of DNA polymerases

Reiner Strick and Charles W. Knopf

Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, D-6900 Heidelberg 1, Germany

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A simple method to assay the major properties of DNA polymerases such as template binding, polymerase and exonuclease activities in one step is exemplified with the DNA polymerases of *E. coli*, bacteriophage T4 and herpes simplex virus. Combining the advantages of the band-shift assay with the resolving power of polyacrylamide gradient gel electrophoresis, the procedure is particularly useful for a rapid functional analysis of mutant polymerases as well as inhibitors of DNA replication.

DNA polymerase; Protein–DNA interaction; Exonucleolytic activity, 3′→5′; Enzymatic assay; Gel electrophoresis

1. INTRODUCTION

Prokaryotic and eukaryotic replicative DNA polymerases share a number of common properties such as (1) binding to the DNA, i.e. to the dNMP primer terminus, in order to initiate the DNA polymerization event, (2) binding to the nucleoside triphosphate, which is needed to extend the polymerisation process, (3) base pair recognition, which is coupled with the dNTP interaction and required for incorporating the correct base, and (4) proof-reading function, a 3′→5′ exonuclease activity, which removes erroneously incorporated and mismatched nucleotides at ongoing polymerization. The exonucleolytic activity, the reversal of the polymerization and the pyrophosphorolysis reaction, occur in the absence of dNTP. But for the latter reaction PP_i has to be present in order that the 3′-terminal nucleotide is released as dNTP (see [1] for review).

Viral DNA polymerases represent successful targets for antiviral chemotherapy, and drugs have been designed to interact in a specific way with the above catalytic activities. We have established an assay method, which at the same time enables us to monitor fate and effect of individual reaction components on both DNA and enzyme during the DNA polymerase–template interaction. Featuring several distinct properties suitable for screening [2,3], the herpes simplex virus type 1 (HSV-1) DNA polymerase was chosen as the test enzyme.

Correspondence address: C.W. Knopf, Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, W-6900 Heidelberg 1, Germany. Fax: (49) (6221) 410700.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonucleases, T4 DNA polymerase, and the Klenow fragment of *E. coli* DNA polymerase I were purchased from Boehringer Mannheim. DNA polymerase of HSV type I strain Angelotti (HSV-1 ANG) was the phosphocellulose fraction prepared as previously described [3]. [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham Buchler (Braunschweig).

2.2. *DNA templates*

The 157 bp *Eco*RI/*Bam*HI restricted HSV-1 ANG DNA fragment derived from plasmid pUC19 into which the *Nae*I fragment of the short repeat region between nucleotide position 555 and 691 [4] was inserted at the *Sma*I site. It was used either unlabeled, or 5′- and 3′-^{32}P-labeled with polynucleotide kinase and Klenow fragment, respectively, using standard procedures [5]. To assure that 3′-^{32}P-labeled fragments contained recessed 3′ ends, a partial filling reaction was performed with Klenow enzyme by using only two nucleotides, 0.5 mM dGTP and [α-^{32}P]dATP.

2.3. *DNA polymerase–DNA binding assay*

DNA polymerase reaction mixtures contained in a final volume of 15 μl: 0.1 μg ^{32}P-labeled *Eco*RI/*Bam*HI fragment (12,000 cpm Cerenkov), 10 mM HEPES/KOH, pH 7.9, 3 mM $MgCl_2$, 50 mM ammonium sulfate, 0.25 mM dithiothreitol, 0.1 mM EDTA, 10% (mass/vol.) glycerol, 100 μg/ml bovine serum albumin, and 0.6 μg HSV DNA polymerase. Incubation was at 25°C for 10 min. To demonstrate the polymerizing activity, reaction mixtures contained unlabeled DNA and the incubation was performed with [α-^{32}P]dATP and the indicated concentrations of dNTP.

2.4. *Band shift electrophoresis*

After adding 3.8 μl loading buffer (0.5 M Tris/borate, pH 8.3, 40% (mass/vol.) glycerol, 50 mM EDTA and 0.25% (mass/vol.) Bromophenol blue), reaction mixtures were loaded onto a 5% (30% acrylamide/0.8% bisacrylamide by mass) native polyacrylamide gel (24 cm × 16 cm × 1.5 mm) or a step-gradient gel, containing a 4 cm bottom layer of 15% polyacrylamide, followed by two layers of 6 and 14 cm in height with 10% and 5% polyacrylamide, respectively. Before loading gels were prerun for 30 min. Electrophoresis was carried out in 0.1 M Tris/borate, pH 8.3, 1 mM EDTA at a constant current of 12 mA

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at 4°C, until the marker dye reached the bottom layer. After electrophoresis the gels were dried onto Whatman paper and exposed to Kodak XAR film overnight.

3. RESULTS AND DISCUSSION

To assess both, DNA binding and catalytic functions of DNA polymerases, in one step, we succeeded to employ a gel retardation assay [6], which was modified as described for optimal DNA binding of the HSV DNA polymerase, used as a test enzyme. In a pilote experiment we found that HSV DNA polymerase bound to the termini of double-stranded DNA, and preferably to DNA with recessed 3'-OH ends, which could be utilized as template for dNTP incorporation. Thus, binding to DNA with blunt ends or single-stranded DNA was more than 20-fold reduced. In order to demonstrate both, the functionality of the enzyme and the specificity of the interaction with the template, reaction mixtures contained either 3'-[^{32}P]dAMP-labeled DNA or unlabeled DNA in combination with [^{32}P]dATP. Fig. 1 shows the result of the subsequent band shift electrophoresis using 5% native polyacrylamide gels. When prelabeled DNA was incubated with HSV polymerase (lane 6) in addition to the band of input DNA (lane 1) two major bands of apparent higher molecular weight were easily detected. These bands resulted from binding of polymerase to DNA as also shown by antibody competition experiments using the recently established monospecific anti-HSV DNA polymerase sera [7; Strick, R., Weisshart, K. and Knopf, C.W., unpublished]. Since the upper band was routinely seen to be more abundant when the enzyme was in excess of the DNA, we propose that the two bands represent DNA molecules with one or two ends being bound by the HSV polymerase. In the presence of labeled triphosphate and with unlabeled DNA in the binding reaction we detected the identical three major bands (lane 2 to 5) as with the prelabeled probe. This result confirmed that the HSV DNA polymerase was functionally active and that a major fraction of the enzyme was still bound to the DNA fragment after the incorporation of label. Also, some extra bands of higher and lower mobility than the input DNA were observed, to some extent probably resulting from the intrinsic exonucleolytic activity of the HSV DNA polymerase [3]. However, if one considers that, in analogy to the Klenow fragment [8], the herpes-viral exonuclease and polymerase activities are similarly organized in separate domains with selective affinities for single- and double-stranded DNA, and that both active sites compete for the 3'-OH primer terminus, then the extra bands of lower mobility than input DNA could reflect alternative conformations of the polymerase–DNA complexes. In two reaction mixtures (lane 4 and 5) unlabeled dGTP was added in order to protect the 3'-terminal dGMP in the *Eco*R1/*Bam*HI cleaved fragment against being hydrolyzed. This treatment seemed to have an influence on the stability of the polymerase–DNA complexes since the input DNA band intensity was considerably reduced. In summary, this first clue experiment demonstrated that HSV DNA polymerase exhibited an affinity to DNA great enough to be analyzed by band shift electrophoresis, and that the enzyme was in fact active under the incubation conditions of the described DNA polymerase–DNA binding assay.

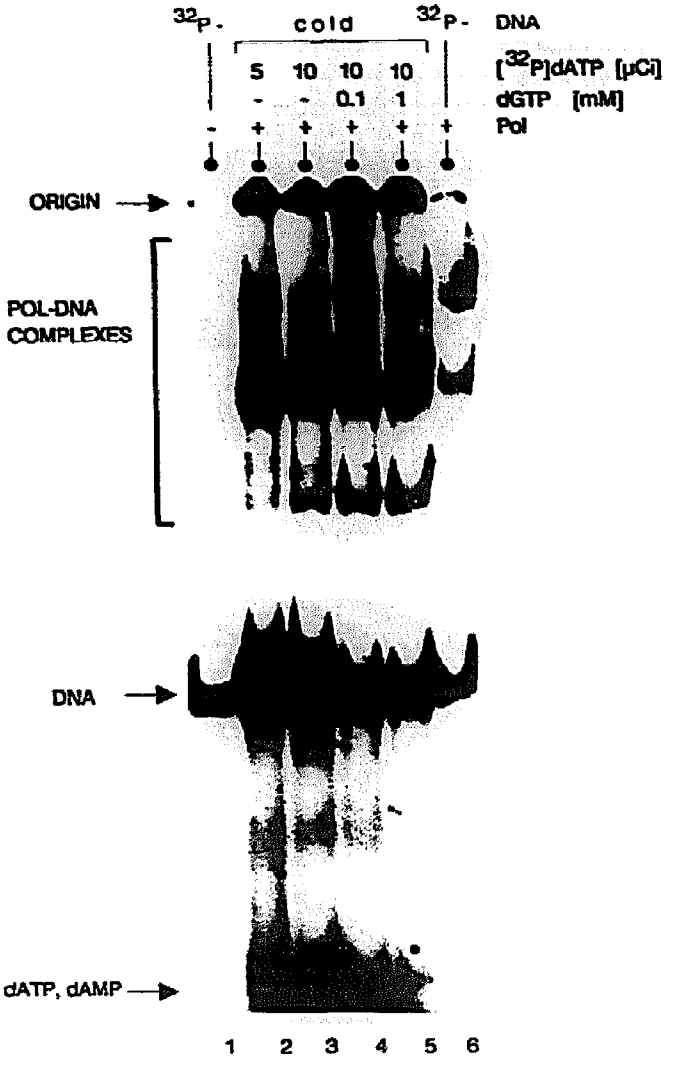

Fig. 1. Band shift electrophoresis of HSV DNA polymerase–DNA complexes formed with 3'-[^{32}P]dAMP-labeled DNA or under self-labeling conditions with cold DNA and in the presence of the indicated triphosphates, respectively, in 5% native polyacrylamide gels. In lanes 2 to 5 one-twentieth of the reaction mixture was loaded onto the gels. The positions of input DNA, polymerase(pol)-DNA complexes and nucleotides are marked.

In a second set of experiments as illustrated in Fig. 2 we have attempted to improve the resolution in the lower molecular weight range by applying a composite native polyacrylamide gel as described. This time 5' and 3' end-labeled DNA was used for the binding reaction, and the influence of PP, examined. With both differently

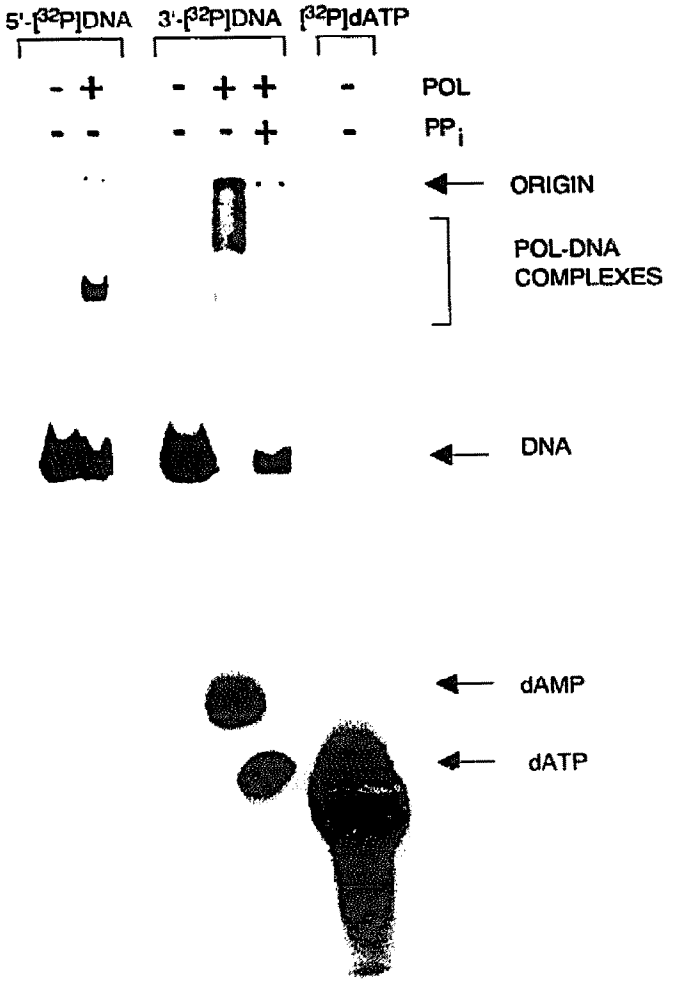

Fig. 2. Assessment of DNA binding, exonucleolytic activity and pyrophosphorolysis reaction by band shift electrophoresis in composite native polyacrylamide gels of DNA polymerase reactions containing HSV DNA polymerase, differently labeled DNA and 5 mM PP_i. [^{32}P]dATP was used as size reference.

labeled DNA probes polymerase–DNA complexes were detected, however the intensities of the label differed. In contrast to the pattern obtained with the 3′ end-labeled probe, no degradation of the 5′ end-labeled DNA was observed, confirming that the employed HSV pol preparation lacked both 5′→3′ exonuclease and endonuclease activities [3]. This result further implied that, for studying the protein–DNA interaction, the template of choice consists of 5′ end-labeled DNA, with one blunt end and one recessed 3′ end, or of a hairpin molecule with only one single-stranded DNA extension. The low molecular weight products generated by the HSV DNA polymerase with the 3′ end-labeled DNA were identified by thin-layer chromatography as well as by including the respective standards in the gel analysis to represent the excised labeled dAMP moiety, which derived from the polymerase-associated 3′→5′ exonuclease. When reaction mixtures contained 5 mM PP_i, no dAMP but a novel even faster migrating spot was found, which was

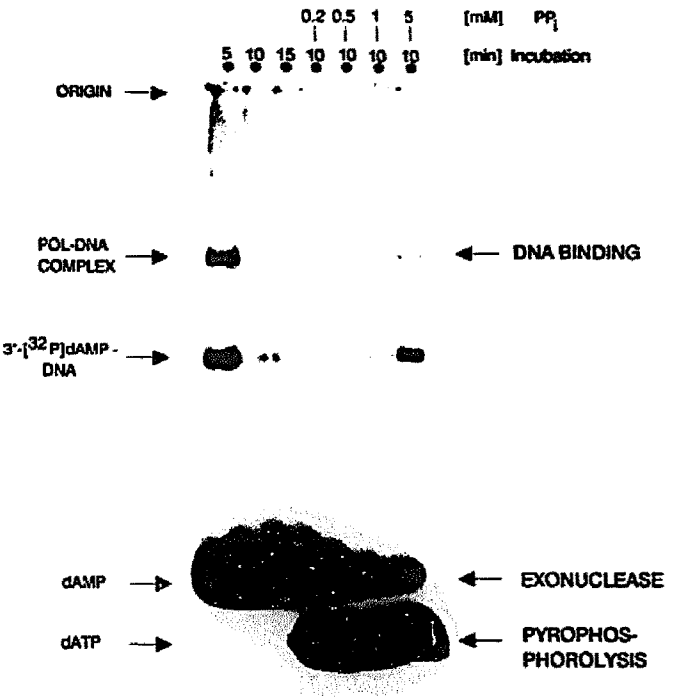

Fig. 3. Simultaneous monitoring of 3′→5′ exonuclease and pyrophosphorolytic activity of HSV DNA polymerase by band shift analysis on a composite gel. Reaction mixtures containing 3′-[^{32}P]dAMP-labeled DNA were incubated with HSV DNA polymerase for the times and with PP_i as indicated. On the left margin the reaction products, and on the right the corresponding polymerase activities are marked by arrows.

identified as [^{32}P]dATP, indicating that the intrinsic exonuclease activity was inhibited, and the reverse reaction of DNA polymerization, the pyrophosphorolysis reaction, was favored (Fig. 2).

The analysis given in Fig. 3 demonstrated that it is generally possible with the employed technique to simultaneously monitor the 3′→5′ exonucleolytic and pyrophosphorolysis activity of the HSV DNA polymerase. Exonuclease activity was apparent by the hydrolysis of dAMP from the input DNA that correlated with the reaction time. In the presence of PP_i the pyrophosphorolysis reaction was clearly discerned from the exonuclease activity by the generation of dATP. Confirming the experiment shown in Fig. 2, the exonucleolytic activity was impaired by higher PP_i concentrations as revealed by the reduction of excised dAMP and by the coincident increase of label in the positions of input DNA and polymerase–DNA complexes.

Naturally, we wanted to see whether the presented method is also applicable to other DNA polymerases. Therefore, commercially available preparations of *E. coli* bacteriophage T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I were tested for comparison as shown in Fig. 4. Binding assays containing 3′ end-labeled DNA were incubated under identical reaction conditions for the stated times

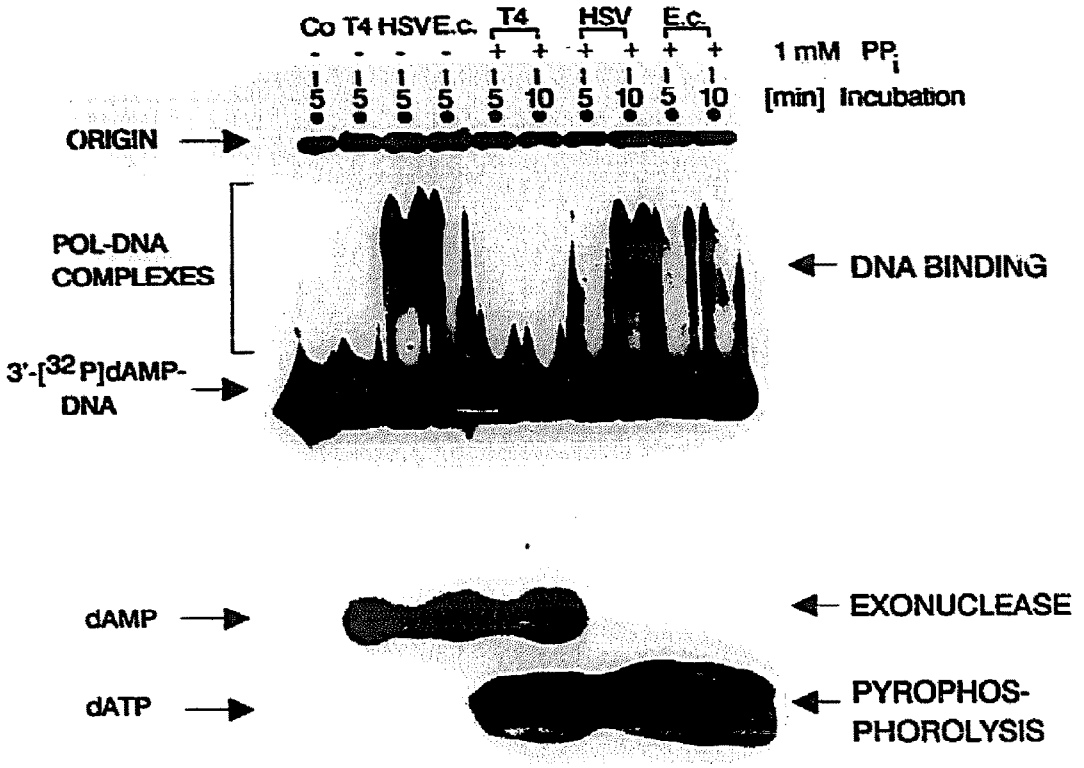

Fig. 4. Comparison of DNA binding, exonuclease and pyrophosphorolysis of the DNA polymerases of HSV, T4 and *E. coli*. Reaction mixtures contained 1 unit of each enzyme and 3'-[^{32}P]dAMP-labeled DNA. Band shift analysis was performed using native composite gels as described. Co, control reaction without enzyme; E.c., *E. coli* Klenow enzyme.

in the absence and in the presence of PP_i. With all three enzymes 3'→5' exonucleolytic as well as pyrophosphorolytic activities were undoubtedly detected. With the T4 DNA polymerase, which displays the strongest 3'→5' exonuclease activity of the compared enzymes [1], neither labeled polymerase–DNA complexes were observed, nor the exonuclease activity was inhibited by 1 mM PP_i. This result correlated quite well with the strength of the T4 DNA polymerase associated exonuclease.

In conclusion, considering that it is possible to vary the polymerase assay with respect to the individual reaction components and to the time for their addition, prior to and after the DNA binding step, the effect of drugs on either of both interactions, at the level of polymerase and nucleic acid binding, can be easily determined. Because all major properties of a DNA polymerase can be conveniently screened by a single gel analysis, another powerful application of the assay is the analysis of mutant polymerases. Furthermore, a quantitative evaluation of the assay is achieved by analyzing the gel right after the run using standard techniques of thin-layer chromatography. In our laboratory the provided protocol is routinely employed to unravel molecular mechanisms of antiviral drug interactions in the target oriented model system.

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