A Sensitive Method for Identification of DNA Dependent DNA Polymerases in Acrylamide Gels after Seperation by Micro Disc Electrophoresis

E. Jürgen Zöllner, Werner E. G. Müller, and Rudolf K. Zahn

Physiologisch-Chemisches Institut, Universität Mainz

(Z. Naturforsch. **28 c**, 376–378 [1973]; received May 9, 1973)

DNA polymerase, disc electrophoresis, template affinity

Two sensitive methods are described for detection of DNA dependent DNA polymerase activities in polyacrylamide gels after their fractionation by micro-disc electrophoresis.

One technique is based on the increase in fluorescence of the ethidium bromide complex with template polydeoxyribonucleotides brought about by the action of the polymerases. The sensitivity of the previously described technique has been enhanced. Another method, 14 fold as sensitive, uses radioactive precursors in the enzyme assay after electrophoretic separation; washing, slicing and counting allows to evaluate incorporation into acid insoluble polymer, requiring $3 \cdot 10^{-2}$ units corresponding to approx. 0.02 μg of *E. coli* DNA polymerase I preparation. Differing activities of polymerase species and differing template preferences may be investigated with these techniques.

Krakow *et al.*[1] and Neuhoff *et al.*[2] described methods how to detect DNA polymerases with disc electrophoresis. Krakow *et al.*[1] used a macro method for the identification of the enzyme activity; a lowest detection level for polymerases corresponding to 1 μg protein in 0.1 ml buffer per gel has been reported[1]. Neuhoff *et al.*[2] could enhance the sensitivity of enzyme detection using a micro disc electrophoresis. This procedure with polyacrylamide gels in capillaries has the great advantage of yielding sharp fractionations in very short times. However, up to now no clearcut correlations between densitometry of treated separation gels, stained with ethidium bromide or pyronine and the covalent incorporation of radioactive deoxyribonucleoside phosphates into DNA could be established. This correlation is reported in this communication applying the sensitive micro disc electrophoresis. The technique with its higher sensitivity may be used as a tool for identification and characterization of polymerases. The determination of their preference for different polydeoxyribonucleotides as templates and primers is possible.

Materials and Methods

Materials were obtained as follows: DNA Dependent DNA polymerase (*E. coli* DNA polymerase I, specific activity: 1800 units/mg), isolated according to the method of Jovin *et al.*[3], DNA dependent DNA polymerase from *E. coli* (specific activity 1200 units/mg), obtained by limited proteolytic action from the *E. coli* DNA polymerase I according to Klenow *et al.*[4], unlabeled deoxyribonucleoside triphosphates, and poly (dA-dT) from Boehringer, Mannheim (Germany); ^{3}H-dTTP (specific activity: 12.2 Ci/mmole) from the Radiochemical Centre, Amersham (England); ethidium bromide from Serva, Heidelberg (Germany); NCS tissue solubilizer from Nuclear Chicago Amersham/Searle Arlington Heights, Illinois (USA) and herring DNA, prepared according to Zahn[5] from H. Mack, Illertissen (Germany).

Electrophoresis was performed according to Ornstein and Davis[6, 7] with the modifications described by Neuhoff[2]. The separating gels of 50 μl were prepared from 10% polyacrylamide with 0.177 M Tris/SO_4^{2-} buffer, pH 8.8; the 8 μl of 5% polyacrylamide spacer gels contained 0.176 M Tris/PO_4^{3-} buffer pH 6.7. Enzymes were applied in 6 μl of 0.05 M Tris/PO_4^{3-} buffer pH 6.7 with 13% sucrose. Electrophoresis was performed at 500 μA per gel of 1.7 mm^2 cross section and a length of 34 mm at 4 °C for 2 hours with the anode at the bottom. The electrophoresis buffer consisted of 6.0 g Tris, 28 g glycin, 3.7 g EDTA per 1000 ml at pH 8.8.

After electrophoresis the gels were incubated for 6 hours at 37 °C in the incubation mixture either as a whole in 2 ml or as small slices in 100 μl each. One incubation mixture contained 50 μg sonicated DNA per ml and dATP, dGTP, dCTP, 0.1 mM each with 0.1 mCi ^{3}H-TTP 40 mM KCl and 6 mM $MgCl_2$ in 20 mM Tris HCl buffer of pH 7.8; the other

Requests for reprints should be sent to Dr. E. J. Zöllner, Physiologisch-Chemisches Institut, Universität Mainz, *D-6500 Mainz*, Johann-Joachim-Becher Weg 13.

mixture contained 10 μg poly (dA-dT) per ml in place of the DNA with the other components unchanged. For detection of the enzymatically synthesized DNA resp. the poly (dA-dT) product, the gels are stained with 100 μg ethidium bromide per ml for 24 hours at 20 °C as described by Krakow *et al.*[1]. After removal of unbound dye, the DNA resp. poly (dA-dT) product/ethidium bromide complex is screened[1] for its fluorescence in ultraviolet light (350 nm) by photographing the gels using B+W 49 ES 1× and 3× filters B+W (Filterfabrik, Wiesbaden), and evaluating the photographs densitometrically as described by us[8]. The incorporation of labeled deoxyribonucleoside triphosphates into acid insoluble material is measured after the electrophoretic run. For this the gels were cut into 1.5 μl slices, incubated for 6 hours as described and then transferred into 1 ml of 5% TCA containing 1% $Na_4P_2O_7$. They were soaked for 1 hour at 4 °C, and washed three times with ice-cold 5% TCA. These gel slices then were heated to 65 °C in 200 μl NCS solution (9 vol NCS/1 vol H_2O) for 1 hour, cooled, quantitatively transferred into 10 ml scintillation liquid, (Hellung-Larson[9, 10]) and counted in a liquid scintillation spectrometer.

Protein was stained with coomasie brillant blue R 250 (C.I. acid blue 83). The amount of enzyme producing the incorporation of 10 nmoles of nucleotides into acid precipitable material within 30 min is taken as one unit of DNA dependent DNA polymerase activity[11].

Results and Discussion

The staining procedure with ethidium bromide[1] is a valuable and very sensitive tool (Figs. 1 and 2) for the detection of polydeoxyribonucleotides, synthesized by the native *E. coli* DNA polymerase I or by its "large fragment", the enzyme A, of Klenow *et al.*[12]. After acrylamide gel micro-disc electrophoresis of the enzymes and incubation of the gels in a reaction mixture containing template and deoxynucleoside triphosphates 0.5 units corresponding to 0.3 μg of the Kornberg-enzyme protein and 0.4 units corresponding to 0.3 μg of its "large fragment" protein can be detected. Thus the resolving power of the technique described here, is higher than reported by Krakow *et al.*[1]. It is in the sensitivity range described by Neuhoff *et al.*[2]. The ethidium bromide complex of the enzymatically synthesized polydeoxyribonucleotide allows for determination with a reproducibility of ±10%. The protein stain pattern of the enzyme in the gels could be correlated

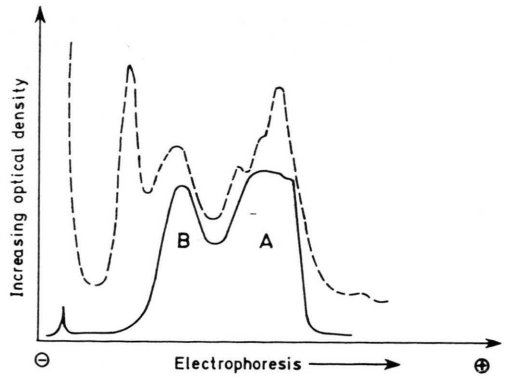

Fig. 1. Densitometry tracing (——) of poly(dA−dT) product, synthesized by *E. coli* DNA dependent DNA polymerase I. 0.5 units were applied per gel and submitted to electrophoresis. The gels then were incubated with a poly (dA−dT) containing reaction mixture and stained with ethidium bromide. The fast running band is marked A, the slow component B. The protein stain of a parallel run with 1.2 μg enzyme protein per gel has been inserted (— — —). See text for details.

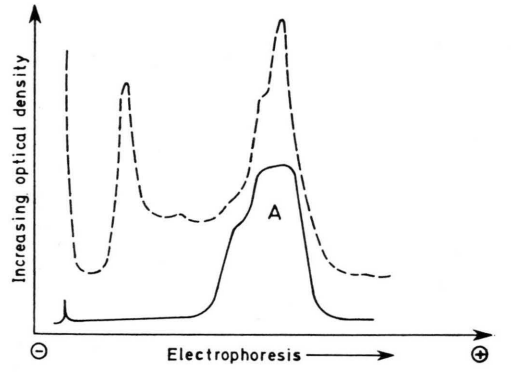

Fig. 2. Densitometry tracing (——) of poly(dA−dT) product, synthesized by 0.4 units of "large fragment" of the DNA dependent DNA polymerase per gel. The protein stain trace for 1.2 μg enzyme protein per gel has been inserted (— — —). For further details see legend to Fig. 1.

quantitatively to the DNA polymerase activity, as shown in Figs. 1 and 2. The micro disc electrophoresis in 70 μl capillaries gives a sharper resolution than the macro-method described[1]. This allows for separation of two separate polymerase activities (Fig. 1) using the purified *E. coli* DNA polymerase I. The slowly migrating band (B in Fig. 1) is homologous to the DNA dependent DNA polymerase whole molecule characterized by a molecular weight of 109,000[13]; the fast band (A in Fig. 1) coincides with the 76,000[13] fragment. This has also been found with phosphocellulose column chromatography[12], and with sodium dodecyl sulfate poly-

acrylamide gel electrophoresis [4, 13]. The ratio of the two polymerase activities can be calculated by densitometric evaluation of the two peaks, caused by the polydeoxyribonucleotides (enzymatically synthesized)/ethidium bromide complexes.

The ratio band A to B (Fig. 1) was 0.73. Thus the technique of micro-disc electrophoresis is applicable for determinations of polymerase activities. For the large fragment of the *E. coli* DNA polymerase I (Fig. 2), reduction to one single activity band could be demonstrated.

In the evaluation of the DNA dependent DNA polymerase activity the incorporation of radioactive labeled nucleoside phosphate into acid insoluble material proved to be more sensitive than the densitometric estimation of the polydeoxynucleotide ethidium bromide complex (Fig. 3).

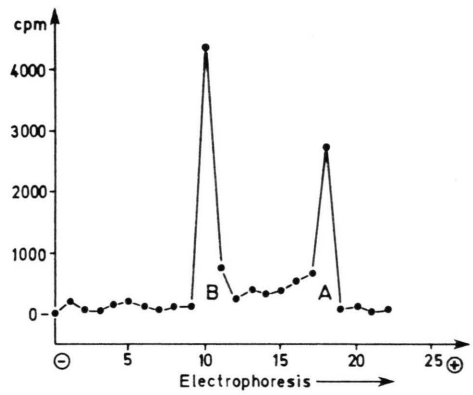

Fig. 3. Distribution of radioactivity after incubating an electrophoretically separated *E. coli* DNA polymerase I preparation in a poly(dA—dT) containing reaction mixture. $3.4 \cdot 10^{-2}$ enzyme units per gel had been applied. Details in the text.

The clear cut separation of the two polymerase moieties in the commercially available *E. coli* DNA polymerase I preparation requires $3.4 \cdot 10^{-2}$ units corresponding to 0.02 μg of protein. This value indicates that this method for detection of the polymerase activity by means of a determination of the incorporated radioactivity is 50 times higher than the one described by Krakow *et al.* [5]. Moreover the resolution of bands with enzyme activity is higher when applying the determination of the incorporated radioactivity than the staining method. The intensity ratio of band A to B of 0.74 corresponds well with the value 0.73 from the ethidium bromide staining technique (Fig. 4). However at higher enzyme concentrations the ratio shifts to higher values possibly due to diffusion phenomenons.

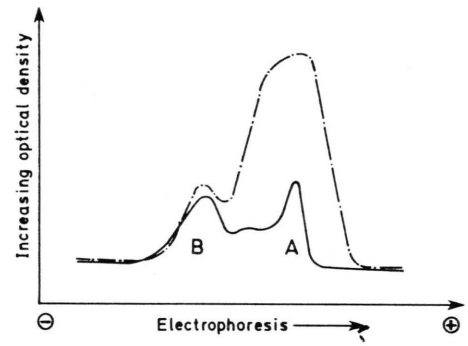

Fig. 4. Densitometry tracing of the polydeoxynucleotide ethidium bromide complex; the polydeoxynucleotide product had been synthesized by *E. coli* DNA polymerase I with poly (dA—dT) (——) or DNA (— · —) in the reaction mixture. 0.2 Enzyme units has been applied per gel. For further details see text and legend to Fig. 1.

Thus a direct correlation of the results for the two methods in the detection of enzymatically synthesized polydeoxyribonucleotides is evident.

The micro-disc electrophoresis technique may also be useful in studies of template preference with bacterial DNA dependent DNA polymerase. As show in Fig. 4 the undigested, whole enzyme (band A) shows higher activity with DNA instead of poly (dA-dT) in the assay mixture, compared to the "large fragment" (band B). This result is in accordance with results presented by Klenow *et al.* [14] obtained with a filter paper technique.

[1] J. S. Krakow and L. D. Murthy, Methods in Enzymology, **Vol. XXI**, Part D, (eds. L. Grossman and K. Moldave), pp. 188—191, Academic Press, New York, London 1971.
[2] V. Neuhoff and A. Lezius, Z. Naturforsch. **23 b**, 812 [1968].
[3] T. M. Jovin, P. T. Englund, and L. L. Bertsch, J. biol. Chemistry **244**, 2996 [1969].
[4] H. Klenow, K. Overgaard-Hansen, and S. A. Patkar, Europ. J. Biochem. **22**, 371 [1971].
[5] R. K. Zahn, E. Tiesler, A. K. Kleinschmidt, and D. Lang, Biochem. Z. **336**, 281 [1962].
[6] L. Ornstein, Ann. New York Acad. Sci. **121**, 321 [1964].
[7] B. I. Davis, Ann. New York Acad. Sci. **121**, 404 [1964].
[8] E. J. Zöllner, W. E. G. Müller, R. K. Zahn, G. Brehm, and G. Korting, Klin. Wschr. **49**, 1290 [1971].
[9] P. Hellung-Larsen, Anal. Biochem. **39**, 454 [1971].
[10] P. Hellung-Larsen, Acta chem. scand. **25**, 1359 [1971].
[11] C. C. Richardson, C. L. Schildkraut, H. V. Aposhian, and A. Kornberg, J. biol. Chemistry **239**, 222 [1964].
[12] H. Klenow and I. Henningsen, Proc. natl. Acad. Sci. USA **65**, 168 [1970].
[13] D. Brutlag, M. R. Atkinson, P. Setlow, and A. Kornberg, Biochem. biophysic. Res. Commun. **37**, 982 [1969].