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

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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 $3 \times 10^{-2}$ units corresponding to approx. $0.02 \mu g$ 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 $1 \mu g$ protein in $0.1 \text{ml}$ 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 $50 \mu l$ were prepared from $10\%$ polyacrylamide with $0.177 M$ Tris/So$_4^{2-}$ buffer, pH 8.8; the $8 \mu l$ of $5\%$ polyacrylamide spacer gels contained $0.176 M$ Tris/PO$_4^{3-}$ buffer pH 6.7. Enzymes were applied in $6 \mu l$ of $0.05 M$ Tris/PO$_4^{3-}$ buffer pH 6.7 with $13\%$ sucrose. Electrophoresis was performed at 500 $\mu A$ per gel of $1.7 \text{mm}^2$ cross section and a length of $34 \text{mm}$ at $4^\circ \text{C}$ for 2 hours with the anode at the bottom. The electrophoresis buffer consisted of $6.0 g$ Tris, $28 g$ glycine, $3.7 g$ EDTA per 1000 ml at pH 8.8.

After electrophoresis the gels were incubated for 6 hours at $37^\circ \text{C}$ in the incubation mixture either as a whole in $2 \text{ml}$ or as small slices in $100 \mu l$ each. One incubation mixture contained $50 \mu g$ sonicated DNA per ml and dATP, dGTP, dCTP, $0.1 \text{mM}$ each with $0.1 \text{mCi} \ ^3 \text{H}-\text{TTP} 40 \text{mM}$ KCl and $6 \text{mM}$ MgCl$_2$ in $20 \text{mM}$ Tris HCl buffer of pH 7.8; the other
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. After removal of unbound dye, the DNA resp. poly (dA-dT) product/ethidium bromide complex is screened 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. 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% Na₄P₂O₇. 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 H₂O) for 1 hour, cooled, quantitatively transferred into 10 ml scintillation liquid, (Hellung-Larson9 '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.

Results and Discussion

The staining procedure with ethidium bromide 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. 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. It is in the sensitivity range described by Neuhoff et al. 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 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. 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; the fast band (A in Fig. 1) coincides with the 76,000 fragment. This has also been found with phosphocellulose column chromatography, and with sodium dodecyl sulfate poly-
acrylamide gel electrophoresis. 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).

For the large fragment of the *E. coli* DNA polymerase I preparation in a poly(dA-dT) containing reaction mixture. 3.4×10⁻² 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 3.4×10⁻² 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. 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.

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. obtained with a filter paper technique.