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Colorimetric Detection of DNA Polymerase Activity after Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis[1]

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A nonradioactive method is developed to detect DNA polymerase activity after sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis containing gapped DNA as template. The technique is based on the use of digoxigenin- or biotin-labeled deoxynucleotides during DNA synthesis, and their detection by means of an anti-digoxigenin antibody–alkaline phosphatase conjugate or by a streptavidin–alkaline phosphatase conjugate. The detection of the DNA polymerase catalytic subunit is achieved after incubation of the gels with colorimetric alkaline phosphatase substrates. The technique is able to detect nanogram amounts of *Escherichia coli* DNA polymerase I and picogram amounts of its Klenow fragment. The results with other DNA polymerases and *E. coli* extracts suggest that this colorimetric detection system could be used for the analysis of an extended range of DNA polymerase enzymes. The method presented in this report offers an alternative to the already described radioactive techniques for detection of DNA polymerase activity after SDS–polyacrylamide gel electrophoresis.

An important objective in the study of an enzyme is the identification of its catalytic subunit. One approach to achieve this objective is the detection of enzymatic activity from its catalytic polypeptide isolated by SDS–PAGE[3] containing the appropriate template or substrate. This technique requires the renaturation of the enzyme and a suitable system to visualize the enzymatic reaction within the polyacrilamide gel. This method, termed "activity gel" analysis, has proven very useful in the investigation of several kinds of proteins (1–4). The first application of this method to the study of DNA polymerases was described by Spanos *et al.* (5). Later, it was shown that the presence of a heterogeneous protein mixture before the denaturation of the enzyme sample (6), or the removal of lipophilic contaminants in SDS detergent after DNA polymerase electrophoresis (7), markedly enhanced the sensitivity of DNA polymerase detection. These modifications enable the detection of homogeneous *Escherichia coli* DNA polymerase I Klenow fragment in the picogram range (6,7). All these studies were based on the use of ^{32}P-labeled deoxynucleotides with high specific activity (5–7).

In the present work, we describe an alternative and efficient nonradioactive method to identify DNA polymerase activity within polyacrylamide gels. The method is based on two commercial nonradioactive-labeled deoxynucleotides used to synthesize DNA probes such as digoxigenin–11-deoxyuridine–triphosphate (8,9) and biotin–14-deoxyadenine–triphosphate (10–12). The results suggest that this nonradioactive technique could be very useful in the investigation of a diverse range of DNA polymerases from homogeneous or heterogeneous samples.

MATERIALS

Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, Tris, glycerol, TEMED, glycine, BSA, DTT, $Mg(CH_3COO)_2$, deoxynucleotide triphosphates, EDTA, NaCl, $MgCl_2$, NBT, BCIP, fetal bovine serum, SDS–molecular weight standard, SDS (product L-4509)

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; BSA, bovine serum albumin; HPM, heterogeneous protein mixture; Tris, tris(hydroxymethyl)aminomethano; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FBS, fetal bovin serum; NBT, nitro blue tetrazolium salt; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Dig-dUTP, digoxigenin-labeled dUTP; Bio-dATP, biotin-labeled dATP; ⟨Dig⟩AP, anti-digoxigenin antibody–alkaline phosphatase conjugate; SA-AP, streptavidin–alkaline phosphatase conjugate.

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E. coli DNA polymerase I Klenow fragment, and *Micrococcus luteus* DNA polymerase were from Sigma. Bromophenol blue was from Bio-Rad Laboratories. Calf thymus DNA and DNaseI were from Worthington Biochemical Corp. The *E. coli* DNA polymerase I was from Pharmacia. *Thermus aquaticus Taq* DNA polymerase, biotin-14-dATP and streptavidin-alkaline phosphatase conjugate were from Gibco-BRL laboratories. The labeled deoxynucleotide digoxigenin-11-dUTP and the anti-digoxigenin-alkaline phosphatase conjugate were from Boehringer-Mannheim Biochemicals.

METHODS

DNA activation. Gapped calf thymus DNA ("activated DNA") was prepared by DNaseI treatment as previously described (13).

DNA polymerase sample preparation. The DNA polymerase samples were prepared according to Karawya *et al.* (6), using different concentrations of BSA or a heterogenous protein mixture (HPM). Fetal bovine serum (FBS) was used as HPM and processed by the following procedure: it was adjusted to 1.2% SDS, 12% glycerol, and 0.7% 2-mercaptoethanol and heated in a boiling water bath for 4 h; it was then concentrated to 55 mg/ml by lyophilization. The DNA polymerase sample (5 μl) was mixed with 10–50 μg of BSA or FBS (2 μl) before the addition of 5 μl of sampling buffer (2.9% SDS, 2.5% 2-mercaptoethanol, 10% (v/v) glycerol, and 0.08 mg/ml bromophenol blue). The amounts of DNA polymerase in the samples are indicated in each experiment. The mixture was heated for 3 min at 37°C and loaded in the gel.

Gel electrophoresis. The SDS–PAGE was prepared following the discontinuous system described by Laemmli (14), adapted to Bio-Rad mini-Protean II dual slab cells (7 × 8 × 0.05 cm). The separating gel contained 7.5% acrylamide, 0.15% bisacrylamide, 0.28 M Tris–HCl, pH 8.8, 0.075% SDS, 0.3 mg/ml ammonium persulfate, 21 μg/ml calf thymus-activated DNA, and 0.2% (v/v) TEMED. Stacking gels were 1 cm high and contained 3% acrylamide, 0.16% bisacrylamide, 0.125 M Tris–HCl, pH 6.8, 0.10% SDS, 0.4 mg/ml ammonium persulfate, and 0.25% (v/v) TEMED. The samples were prepared as described above, loaded in 0.5-mm wells, and electrophoresed at a constant voltage of 100 V until the tracking dye reached the bottom of the separating gel, about 1–1.5 h at 4°C.

DNA polymerase renaturation and enzyme assay. After electrophoresis, the gel was washed twice in 50 ml of 50 mM Tris–HCl, pH 7.5, for 15 min at room temperature with gentle agitation. Each gel was then incubated for 3 h in 40 ml of renaturation buffer (50 mM Tris–HCl, pH 8.0, 10 mM 2-mercaptoethanol, 10 mM $Mg(CH_3COO)_2$, 400 μg/ml BSA, 0.4 mM EDTA, and 16% (v/v) glycerol), under the same conditions described above. After the renaturation of the enzymes, each gel was rinsed three times in 50 ml of 50 mM Tris–HCl, pH 8.0. Depending on the labeling system used, each gel was incubated for 20 h at 37°C without agitation in 25 ml of either Mix-B buffer (50 mM Tris–HCl, pH 8.0, 10 mM DTT, 10 mM $Mg(CH_3COO)_2$, 400 μg/ml BSA, 0.4 mM EDTA, and 16% (v/v) glycerol, 10–44 nM Bio-dATP, 10 μM each dCTP, dGTP, and dTTP) or Mix-D buffer (50 mM Tris–HCl, pH 8.0, 10 mM DTT, 10 mM magnesium acetate, 400 μg/ml BSA, 0.4 mM EDTA, 16% (v/v) glycerol, 10–44 nM Dig-dUTP, 10 μM each dATP, dCTP, and dGTP) or as indicated for each experiment.

Developing the colorimetric reaction. After removal of the reaction buffer, Mix-B or Mix-D, the gels were rinsed briefly three times with several volumes of TN buffer (0.1 M Tris–HCl, pH 7.5, and 0.15 M NaCl). The gels were then blocked for 1 h with 4% nonfat milk in TN buffer, rinsed three times with TN buffer, and incubated for 10–15 h in TN buffer containing either 0.75 U/ml of anti-digoxigenin antibody–alkaline phosphatase conjugate (⟨Dig⟩AP) or 0.1 μg/ml of streptavidin–alkaline phosphatase conjugate (SA-AP). These conjugate concentrations correspond to dilutions of about 1:5000 with respect to the commercial solutions (8,10). The gels were then rinsed three times with AP 9.5 buffer (0.1 M Tris–HCl, pH 9.5, 5 mM $MgCl_2$, and 0.1 M NaCl) for 20 min. All the above procedures were performed with gentle agitation at room temperature. To develop the colorimetric assay by means of the alkaline phosphatase conjugates, each gel was immersed in buffer AP 9.5 containing 100 μg/ml NBT and 60 μg/ml BCIP. The visualization of the DNA polymerase bands was carried out after 1–4 days of incubation in the dark at room temperature without agitation. The alkaline phosphatase reaction was stopped by washing the gels three times with stop buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA); gels were then dried between two cellophane papers and photographed.

Preparation of the E. coli crude extracts. A total of 1.4 g of bacteria was resuspended in 5 ml PBS (10 mM sodium phosphate, ph 7.2, 0.15 M NaCl), sonicated by five cycles of 30 s at 200 W, and centrifuged at 10,000*g* for 10 min. The supernatant was used to detect DNA polymerase activity. All the procedures were carried out at 0–4°C.

The MW-SDS-200 Sigma kit of molecular weight standards contained the following proteins: carbonic anhydrase from bovine erythrocytes (29,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase B from rabbit muscle (97,400), β-galactosidase from *E. coli* (116,000), and myosin from rabbit muscle (205,000).

RESULTS AND DISCUSSION

To detect DNA polymerase activity by means of this nonradioactive method, the samples were prepared according to the improved conditions described by Karawya *et al.* (6). Prior to electrophoresis, the DNA poly-

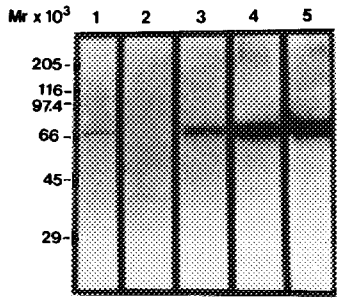

FIG. 1. Effect of the biotin-dATP concentration on the colorimetric detection of *E. coli* DNA polymerase Klenow fragment. Samples with 0.3 units (equivalent to 22 ng) of enzyme were mixed with 11 μg of FBS. After electrophoresis, the gel was cut into sections which were individually incubated with reaction buffer Mix-B containing 0, 1, 10, and 20 nM of Bio-dATP (lanes 2–5). Well 1 contains 0.3 μg of Klenow fragment stained with Coomassie blue. All the procedures were performed as described under Methods except as indicated on the figure.

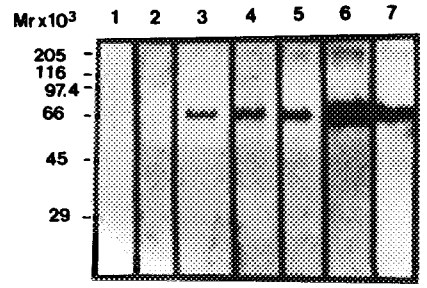

FIG. 2. Effect of different incubation times on the assay of DNA polymerase activity linked to the gel matrix. Samples of 0.3 units of *E. coli* DNA polymerase Klenow fragment were mixed with 11 μg of FBS. After electrophoresis, the gel was cut into sections which were separately incubated for 0, 1, 2, 10, 22, and 50 h (lanes 2–7), in a reaction buffer Mix-B in which the concentrations of Bio-dATP and dATP were 10 and 24 nM, respectively. Well 1 contains 0.3 μg of Klenow fragment stained with Coomassie blue. All the procedures were performed as described under Methods except as indicated on the figure.

merase samples were mixed with either BSA or FBS. Nevertheless, too high concentrations of BSA or FBS produced dispersion and aberrant migration of the DNA polymerase samples. To obtain the best signal, the optimal amount of BSA or FBS in each sample was 10–20 μg (data not shown).

To study the dependence of signal intensity upon the concentration of the labeled deoxynucleotide, the experiment presented in Fig. 1 was performed. A single band is observed corresponding to M_r 68,000 which is strongly dependent on the labeled deoxynucleotide concentration. This molecular weight agrees with that described for the *E. coli* DNA polymerase Klenow fragment (5,15). This figure also shows that it is possible to obtain a good colorimetric signal with concentrations as low as 1 nM of labeled deoxynucleotide substrate (lane 3). These results eliminate the possibility that the signal is caused by a mechanism independent of DNA polymerase activity, such as contamination with alkaline phosphatase or nonspecific binding of streptavidin conjugate to protein samples within the gel matrix. On the other hand, similar to what Spanos *et al.* described (5), the highest levels of DNA polymerase activity were achieved using very little or even none of the nonlabeled counterpart dATP. The same situation was observed with Dig-dUTP.

The time course of the DNA polymerase reaction linked to the gel matrix is shown in Fig. 2. The optimal incubation time was 22 h, similar to that found for radioactive techniques (5,6).

Figure 3 shows that both the digoxigenin and the biotin systems allow the detection of 2 ng of *E. coli* DNA polymerase I and at least 20 pg of Klenow fragment. This sensitivity is equivalent to that described for radioactive methods (6,7) and it could be increased by elevating the labeled deoxynucleotide concentration to micromolar levels, similar to the conditions used with the radioactive techniques. Also, under these conditions, it can be observed that the DNA polymerase activity bands detected by the digoxigenin system are more intense than those obtained with the biotin system. Nevertheless, this difference could be due not only to the dissimilar nature of the two labeled deoxynucleotides, but also to other causes, such as the nature of the two types of alkaline phosphatase conjugates or the deoxynucleotide composition of the activated DNA used as template.

The results illustrated in Fig. 4 show that this nonradioactive technique can detect DNA polymerases other than *E. coli* DNA polymerase I and its Klenow fragment. In lane 3, two major bands were observed with M_r about 83,000 and 97,000 corresponding to a *Taq* DNA polymerase sample (16). Lane 4 showed bands with M_r 70,000 and 105,000 which corresponded to a *M. luteus* DNA polymerase sample (17,18). Apparently, the detection efficiency for other enzymes is lower than that observed with the Klenow fragment. It is possible that this differ-

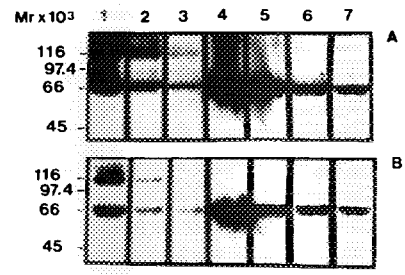

FIG. 3. Sensitivity of the colorimetric detection of DNA polymerase activity by means of digoxigenin-dUTP (gel A) or biotin-dATP (gel B) as labeled enzyme sustrate. Samples with 195, 19.5, and 2 ng of *E. coli* DNA polymerase I (lanes 1–3) or 22, 2.2, 0.22, and 0.02 ng of Klenow fragment (lanes 4–7) were mixed with 11 μg of FBS. Gel A was incubated in reaction buffer Mix-D in which the concentration of Dig-dUTP was 20 nM. Gel B was incubated in reaction buffer Mix-B in which the concentration of Bio-dATP was 20 nM. All the procedures were performed as described under Methods except as indicated on the figure.

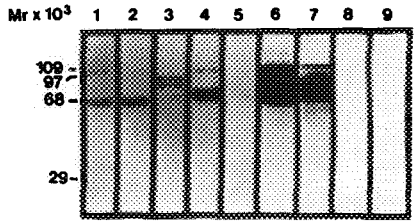

FIG. 4. Colorimetric detection of DNA polymerase activity from different sources using digoxigenin-dUTP. A single gel was loaded with 0.046 units of *E. coli* DNA polymerase I (lane 1), 0.003 units of Klenow fragment (lane 2), 0.25 units of *Taq* DNA polymerase (lane 3), 0.017 units of *M. luteus* DNA polymerase (lane 4), and 5 μl of *E. coli* extract (lanes 6–9). All samples (except lanes 7 and 9) were mixed with 11 μg of FBS. The gel was cut into two sections which were incubated in reaction buffer Mix-D without Dig-dUTP (lanes 8 and 9) or with 40 nM of Dig-dUTP (lanes 1–7). All the procedures were performed as described under Methods except as indicated on the figure.

ence could be explained by the capacity of each DNA polymerase type to renaturate the active conformation after SDS treatment (1), or due to the specific catalytic properties of each enzyme (19,20). Hence, these observations strengthen the idea that to obtain the highest sensitivity it is necessary to determine the best assay condition for each type of DNA polymerase.

To investigate if this colorimetric technique could be used for the detection of DNA polymerases from heterogeous samples, *E. coli* extracts were analyzed with this activity gel technique (Fig. 4). Similar electrophoretic patterns, with several bands between M_r 68,000 and 109,000, were observed in *E. coli* extracts with (lane 6) and without (lane 7) the addition of FBS protein mixture. This electrophoretic pattern is analogous to other patterns obtained with radioactive techniques in which, the 68,000 band probably corresponds to the Klenow fragment and the 109,000 band corresponds to the intact *E. coli* DNA polymerase I (5,7). No colorimetric signals are observed in lanes 8 and 9, where the same *E. coli* extract was assayed without the digoxigenin-dUTP substrate. These results discard any possibility that the colorimetric signals could be due to the endogenous alkaline phosphatase enzymes or nonspecific binding of the alkaline phosphatase conjugate.

Therefore, this experiment show that these colorimetric techniques may be used to detect DNA polymerase enzymes in a heterogenous sample such as an *E. coli* crude extract. Nevertheless, it is necessary to eliminate the possibility that some bands could be due to endogenous alkaline phosphatase activity. This objective can be obtained by the heat inactivation of this enzyme (21) or by performing adequate negative controls, such as the omission of the labeled deoxynucleotide.

All the experimental procedures of the colorimetric technique could be performed within 3 days and the DNA polymerase bands could be visualized after 1 to 4 days. These time periods are similar to those indicated for radioactive techniques, although the detection of radioactive bands may be faster (6,7). However, in some cases depending upon the amount or the nature of the sample, the detection of the radioactive signals needs several days of autoradiography (7). On the other hand, the colorimetric technique is less expensive than the radioactive technique. This is based upon the amount of radioactivity of ^{32}P-labeled deoxynucleotide used in each determination, which varies between 250 and 80 μCi for each activity gel experiment (6,7), while only 0.25–1 nmol of the nonradioactive-labeled deoxynucleotides are necessary to obtain good DNA polymerase signals and high dilutions of the alkaline phosphatase conjugates. Finally, the colorimetric technique is a harmless method that does not need the rigorous safety conditions established for working with radioactive materials.

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