A Non-radioactive, Zymographical Method for Detecting Mammalian DNA Repair Enzymes

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ABSTRACT. A non-radioactive zymographical method for detecting mammalian DNA repair enzymes is presented. The method consists of the following steps: (1) preparations of crude, partially purified enzymes; (2) SDS-(denatured) polyacrylamide gel electrophoresis (PAGE) of the enzyme preparations; (3) renaturation of proteins electrophoresed on SDS-PAGE; (4) preparation of damaged DNA-fixed membranes; (5) protein blotting (activity blotting) onto a damaged DNA-fixed membrane, a process during which incision and/or excision are introduced to the damaged DNA by a repair enzyme(s); (6) non-radioactive detection of the activity-blotted site(s) to localize the repair enzyme. The present technique was developed using mouse APEX nuclease, a multifunctional DNA repair enzyme, and a DNA-fixed membrane treated with bleomycin or acid-depurinated, and was applied for the detection of human liver APEX nuclease in partially purified preparations. The sites primed on damaged DNA by APEX nuclease, which has priming activity for DNA polymerase on bleomycin-damaged DNA and acid-depurinated DNA produced by generating free 3′-hydroxyl termini, were labeled with digoxygenin-11-dUTP by incubation with a digoxygenin-labeled substrate and Klenow polymerase. The digoxygenin-labeled DNA on the activity-blotted membrane was detected by a sensitive chemiluminescent reaction. Human liver APEX nuclease in a partially purified preparation and an active peptide possibly derived from the enzyme were clearly demonstrated by the present method.

Key words: non-radioactive zymographical detection — activity blotting — mammalian APEX nuclease — human liver APEX

Various types and unexpectedly large amount of DNA damage occur in cells. The DNA lesions are generally repaired to their original state by cellular DNA repair systems.[1-3] However, the repair mechanisms occasionally fail, resulting in genetic changes or cell death. These genetic changes are the possible causes of cellular transformation, aging, molecular diseases and genetic diversity.[1,4,5] To understand the mechanisms of genetic maintenance and their relation to genetic changes, knowledge of DNA repair enzymes is very important. DNA excision repair occurs as a result of the following sequential reactions: priming (recognition of DNA damage and an incision and/or excision reaction), repair DNA synthesis, repair patch ligation, and restoration of chromatin structure. The factors involved in the priming steps are complicated because of the variety of DNA damage. Several tens or more

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enzymes have been suggested to be involved in the priming steps, although only a few of them have been identified.

A zymographical procedure, the activity blotting method, has been developed to detect DNA-modifying enzymes.[6-10] This method has been shown to be very useful for molecular-mass estimation, characterization (or identification) and purification of the DNA repair enzymes involved in the priming steps. In the activity blotting method used prior to this one, and which is still in use, the zymography to localize the target priming enzymes has been performed by autoradiography.[6-9]

The aim of the present experiment was to establish a non-radioactive, activity blotting method for detecting mammalian DNA repair enzymes. This non-radioactive procedure should increase the applicability of the method and should be safe not only for the person involved in the experiments but also for the environment. To extend the application of the non-radioactive method to the examination of clinical specimens, the method was successfully applied for detection of human liver APEX nuclease partially purified from the liver specimen of an autopsy.

MATERIALS AND METHODS

Materials

A Klenow fragment (Klenow polymerase) of *E. coli* DNA polymerase I was purchased from Takara Shuzo Co. Ltd., Kyoto. Highly polymerized calf thymus DNA was obtained from Pharmacia LKB Biotechnology. Nylon membrane (Hybond-N$^-$) was purchased from Amersham. Digoxygenin-labeled dUTP and a DIG luminescent detection kit were obtained from Boehringer Mannheim. Reagents for electrophoresis were purchased from Nakarai Tesque, Inc., Kyoto. Mouse ascites sarcoma (SR-C3H/He) cells were obtained and maintained as has been described previously.[11] Mouse DNA polymerase was purified from SR-C3H/He cells as described previously.[12] About 50 g of human liver tissue was obtained two hours postmortem from an autopsy case of pulmonary carcinoma. The other reagents used were obtained as has been described by other authors previously.[13]

Preparation of damaged DNA-fixed membranes

The stock solution of DNA (2.5 mg/ml in deionized water) was diluted with two-fold-concentrated standard saline citrate (2×SSC; 1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to a final concentration of 0.145 mg/ml. A Nylon membrane (6×9 cm) prewetted in 2×SSC was immersed in the DNA solution in a plastic beaker or in a sealable plastic bag, and incubated with gentle movement of the solution at room temperature for 1 h. After the incubation, the membrane was rinsed three times in 2×SSC, blotted on Kimwipe or a coarse filter paper to remove excess fluid, and then air-dried overnight in the dark. The native DNA-fixed membrane was stored in a dark box until use.

The bleomycin-damaged DNA-fixed membrane was prepared by treating the native DNA-fixed membrane at 37°C for 30 min with Triton-buffer B (0.0175% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) supplemented with 5 μg/ml

bleomycin A_2 and 30 μM ferrous ammonium sulfate.[6,13] To prepare an acid-depurinated DNA-fixed membrane, the native DNA-fixed membrane was incubated in 37.5 mM sodium citrate (pH 3.5) at 60°C for 30 min.[6,13] The acid-depurination of DNA was conducted just before activity blotting to avoid spontaneous nicking. After the treatment with DNA-damaging agents, the membranes were rinsed three times in 2×SSC, rinsed three times in a blotting buffer (40 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.02% sodium azide and 6 mM 2-mercaptoethanol), and used for protein blotting.

Extraction and partial purification of a mammalian repair enzyme, APEX nuclease

Mammalian major AP endonuclease (designated as APEX nuclease) was extracted from permeabilized mouse ascites sarcoma cells or human liver tissue with 0.2 M potassium phosphate (KPi) buffer (pH 7.5), as has been described previously.[7,12,14] After adjustment of the potassium phosphate concentration to 0.1 M, the extract (F_1 fraction) was passed-through a DEAE-cellulose column conditioned with 0.1 M KPi buffer to remove nucleic acids. The passed through sample (F_2 fraction) was loaded onto a phosphocellulose column equilibrated with 0.1 M KPi buffer. The enzymens adsorbed on phospho-cellulose were eluted with 0.1 M KPi buffer. The enzymes adsorbed on phosphocellulose were with 0.3 M KPi buffer. The eluent was diluted with deionized water to 0.1 M potassium phosphate or dialyzed against 50 mM Tris-HCl and 1 mM EDTA, pH 8.0, to reduce the salt concentration, because electrophoresis of proteins on SDS-polyacrylamide gels is disturbed when the ionic strength of the sample buffer is high. The phosphocellulose fraction (fraction F_3) concentrated by ultrafiltration was mixed with a one fourth volume of the four-fold concentrated gel loading buffer for SDS-PAGE (see below).[12,15] The mixture in a Eppendorf tube was immersed in a boiling water bath for 2 min, and after chilling, an aliquot of the mixture was applied to SDS-PAGE.

Preparation of *E. coli* whole cell extract

E. coli HB101 strain cells were suspended in 1 ml of the loading buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).[15] The cell suspension was boiled for 5 min and then immediately centrifuged at 10,000 g for 10 min. The supernatant was used as *E. coli* whole cell extracts for application to SDS-polyacrylamide gels.

SDS-polyacrylamide gel electrophoresis

Laemmli's buffer system[15] was used for SDS-PAGE with minor modifications and a 8×8.5×0.1 cm slab gel was used for the activity blotting method. The electrode buffer contained 0.025 M Tris-base, 0.192 M glycine, and 0.1% SDS, pH 8.3. The stacking gel contained 3% acrylamide, 0.08% *N, N'*-methylenebisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% *N,N,N', N'*-tetramethylethylenediamine (TEMED), and 0.03% ammonium persulfate. The separation gel contained 12% acrylamide, 0.33% *N,N'*-methylenebisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% TEMED, and 0.05% ammonium persulfate. The samples in the gel loading buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 2.5% 2-mercaptoethanol, 0.25 M sucrose, and

0.01% bromphenol blue) were incubated at 100°C for 2 min, and loaded onto the gel. Electrophoresis was conducted at room temperature at a constant current of 15–20 mA until the dye front reached 2 mm over the bottom of the gel (approximately 2–3 h). The Molecular mass marker used for SDS gel electrophoresis was MW-SDS-70L, Sigma Chemical.

Renaturation of enzymes in SDS-polyacrylamide gel

After electrophoresis the gel was rinsed briefly in a renaturation buffer consisting of 40 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.02% sodium azide and 0.1% (w/v) Triton X-100, and shaken in the buffer at 4°C for 2–3 h with three changes of the buffer. The gel was left overnight at 4°C in fresh buffer with gentle shaking. Then it was washed at room temperature three times for 20 min each with the blotting buffer (40 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.02% sodium azide and 6 mM 2-mercaptoethanol).

The blotting of enzyme activity from gels on damaged DNA-fixed membranes was performed mainly by the capillary blotting method and were compared with results obtained by the method of electroblotting method.

Activity blotting to damaged DNA-fixed membranes by capillary blotting

Capillary transfer of enzyme activity from the renatured gel to a damaged DNA-fixed Nylon membrane was performed essentially as described for DNA transfer by Southern[16,17] with the following modifications. The renatured gel was placed on a transfer support covered with Whatman 3 MM paper. The damaged DNA-fixed membrane was then placed onto the gel, and proteins (activities) were transferred to the membrane with the blotting buffer at 30°C for 24–48 h. Incision and/or excision (or DNA modification) by the enzymes occurred during the blotting process. After the blotting, the membrane was washed for 20 min with three changes of TEN buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl). After the washing, the membrane was proceeded directly to the next detection step.

Protein blotting to damaged DNA-fixed membranes by electroblotting

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to a damaged DNA-fixed membrane was performed by the Western blotting technique[17,18] with the following modifications. After electrophoresis, the gels were preincubated at 37°C for 20 min in a reduction buffer containing 7 mM 2-mercaptoethanol, 192 mM glycine and 25 mM Tris-HCl, pH 8.3, with constant shaking. The incubation was repeated twice with the fresh reduction buffer. Electrotransfer of proteins onto a damaged DNA-fixed membrane was performed at 0°C, 70 V for 1 h. The electroblotted membrane was rinsed three times and soaked with gentle shaking at 4°C overnight in a renaturation buffer consisting of 40 mM Tris-HCl, pH 8.0, at 25°C, 2 mM $MgCl_2$, 0.02% sodium azide and 0.1% Triton X-100. The membrane was incubated at 37°C for 30 min in the same buffer to prime for DNA synthesis (the blotted enzyme incises and/or excises the damaged DNA fixed on the membrane). The membrane was washed three times for 20 min each with TEN buffer. After the washing, the membrane was proceeded directly to the next detection step.

Localization of DNA repair enzymes

The procedures for demonstrating the target enzyme on the activity-blotted membrane varied with the enzyme function and the damaged DNA fixed on the membrane. In the present experiments, *E. coli* exonuclease III and APEX nuclease, a mammalian major AP endonuclease, were selected to demonstrate the activity blotting. These enzymes have priming activity for DNA polymerase on bleomycin-damaged and acid-depurinated DNAs by generating free 3'-hydroxyl termini.

The activity-blotted membrane was incubated with 3% BSA in TEN buffer at 37°C for 1 h to saturate remaining protein binding sites and then was washed three times for 5 min each with a buffer (40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and 50 mM NaCl). The sites primed by the activity blotting were labeled with digoxygenin-dUTP by DNA synthesis under the presence of the digoxygenin-labeled substrate. The incorporated digoxygenin-dUTP was detected using the DIG luminescent detection kit according to the directions for users of Boehringer Mannheim. Briefly, the damaged DNA-coated membrane primed by activity blotting and labeled with digoxygenin-dUTP was incubated with antidigoxygenin Fab fragments conjugated to alkaline phosphatase to induce immunological binding between the antibody-conjugate and digoxygenin-dUTP-labeled DNA. The membrane having the antibody hapten complex was incubated with AMPPD [3-(2'spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-pheny-1,2-oxetane], an alkaline phosphatase substrate. The luminescence produced by the alkaline phosphatase-catalyzed reaction was detected on X-ray film. Protein concentrations were determined by a dye-binding method (Bio-Rad).

RESULTS AND DISCUSSION

Non-radioactive, zymographical detection of mouse APEX nuclease and *E. coli* exonuclease III

Apurinic/apyrimidinic (AP) sites are generated in DNA by spontaneous hydrolysis, radiation, oxidative damage and the action of DNA glycosylases removing bases modified by various DNA damaging agents.[1-3] The AP sites are the most frequent lesions found in cellular DNA and amount to over 10,000 residues/mammalian cell per day.[2] Single-strand breaks with 3' termini blocked with nucleotide fragments are also produced by free radical pathways caused by ionizing radiation, bleomycin or other sources of oxygen radicals. These lesions are known to occasionally induce cytotoxic and mutagenic effects. The APEX nuclease and exonuclease III used as model enzymes in the present experiments for establishing a non-radioactive zymographical method are thought to be involved in the repair of such lesions.[1,2,7,8,12,19]

APEX nuclease is known to be a mammalian homolog of *E. coli* exonuclease III.[7,8] Both enzymes are multifunctional enzymes having 5' AP endonuclease, 3'-5' exonuclease, DNA 3' repair diesterase and DNA 3'-phosphatase activities.[1,2,7,8,12,19] The DNA 3' repair diesterase activity of these enzymes (APEX nuclease and exonuclease III) acts as a priming enzyme for DNA polymerase on bleomycin-damaged DNA by removing 3' phosphoglycolate termini and restoring free hydroxyl termini. The 5' AP endonuclease activity of these enzymes acts as a priming enzyme for DNA polymerase on

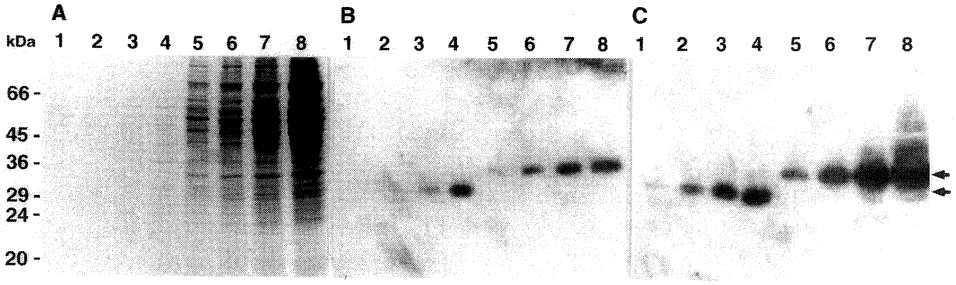

Fig. 1. Non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities by the capillary blotting procedure of the activity blotting method. The *E. coli* HB101 strain cell extract and the partially purified preparation (F3 fraction) of mouse APEX nuclease were prepared and electrophoresed on SDS-polyacrylamide gels as described in MATERIALS AND METHODS. A, the CBB-stained gel. B, proteins of the renatured gel were capillary-blotted on bleomycin-damaged DNA-fixed membrane. C, proteins of the renatured gel were capillary-blotted on acid-depurinated DNA-fixed membrane. The membranes were incubated for DNA synthesis and the sites of substrate incorporation (primed sites) on the membranes were visualized by the digoxygenin luminescent detection. Amount of the *E. coli* cell extract loaded; 0.1 μg in lane 1, 0.2 μg in lane 2, 0.4 μg in lane 3, 0.8 μg in lane 4; amount of the F_3 fraction, 8.8 μg in lane 5, 17.5 μg in lane 6, 35 μg in lane 7 and 70 μg in lane 8. The positions indicated by the right upper and lower arrows are the bands of APEX nuclease and exonuclease III, respectively.

acid-depurinated (AP) DNA by introducing single-strand nick at the 5′ side of AP sites and producing 3′ hydroxyl termini.

Fig. 1 shows the result of non-radioactive detection after the capillary blotting of activities of partially purified mouse APEX nuclease and *E. coli* whole cell extract containing exonuclease III. The bleomycin-damaged DNA-fixed membrane is used for the detection of DNA 3′ repair diesterase activity which is involved in the conversion of the 3′-phosphoglycolate termini (3′-blocked DNA damage) of bleomycin-damaged DNA into free 3′ hydroxyl termini. The acid-depurinated DNA-fixed membrane is used for the detection of 5′ AP endonuclease activity. The 3′-hydroxyl termini produced by either DNA 3′ repair diesterase activity or 5′ AP endonuclease activity provide primed sites for DNA polymerase, which is included in the detection system of the present activity blotting. The result shown in Fig. 1 indicates that about 30 kDa protein in the *E. coli* extract and about 35 kDa protein in the partially purified mouse APEX nuclease have priming activity on both the bleomycin-damaged DNA- and acid-depurinated DNA-fixed membrane. This means that these *E. coli* 30 kDa and mouse 35 kDa proteins have DNA 3′ repair diesterase and 5′ AP endonuclease activities. The known enzymes having these activities and molecular mass numbers (30 kDa and 35 kDa) are exonuclease III in *E. coli* and APEX nuclease in mammalian calls. The signal intensities are semi-dose dependent. Comparing these results with those of the activity blotting method established using the radioactive detection method,[6-8] the non-radioactive detection method is more convenient than the radioactive one, although the sensitivity of the former is slightly lower than that of the latter. Taken all together, the non-radioactive method bears comparison with the radioactive one.

The electroblotting method is principally quite different from that of the capillary method. In electroblotting, proteins are effectively blotted on the damaged DNA-fixed membrane, but the priming for DNA polymerase on the acid-depurinated or bleomycin-damaged DNA by APEX nuclease and exonuclease III dose not occur during the blotting process, because Mg^{2+} ion, which is essential for the priming reactions, precipitates on the electrode as a salt during the electrophoresis. Therefore, it can not be added to the blotting buffer. To prime for DNA synthesis, proteins should be renatured after the electroblotting and the protein-blotted, damaged DNA-fixed membrane must be incubated at 37°C for 30 min. In comparison with this, the priming by the enzymes occurs fairly well during the process of capillary blotting, although protein blotting on the damaged DNA-fixed membrane is incomplete.

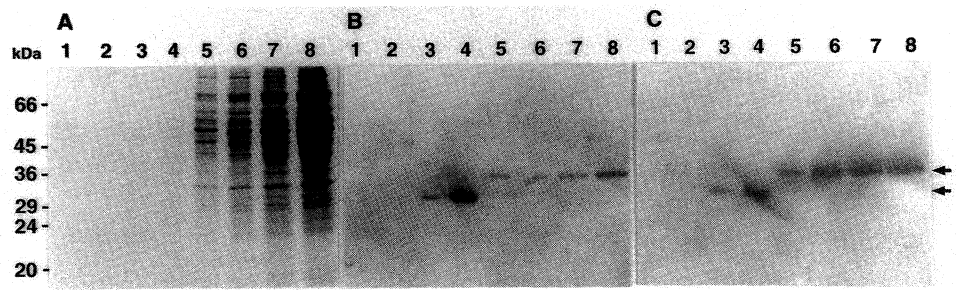

Fig. 2. Non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities by the electroblotting procedure of the activity blotting method. The *E. coli* cell extract (lanes 1–4) and the F_3 fraction (lanes 5–8) of mouse cell extract were electrophoresed on SDS-polyacrylamide gels. Fractionated proteins were stained with CBB in (A). Fractionated proteins were electroblotted on a bleomycin-damaged DNA-fixed membrane (B) or an acid-depurinated DNA-fixed membrane (C). The membranes were washed and soaked in the renaturation buffer, and then incubated at 37°C for 30 min in the same buffer to prime for DNA synthesis. The incubation for DNA synthesis and the chemiluminescent detection of the primed site were performed as described in MATERIALS AND METHODS. The sample and its amount loaded in each lane were the same as described in the legend to Fig. 1.

Fig. 2 shows the results of non-radioactive detection after the electroblotting of activities of the partially purified mouse APEX nuclease and the *E. coli* whole cell extract containing exonuclease III. The detection sensitivities of the electroblotting were generally lower than those of the capillary blotting, although the processing time was shorter in the former than in the latter.

Detection of human liver APEX nuclease by the activity blotting method

Human liver APEX nuclease was extracted and partially purified by sequential chromatographies with DEAE-cellulose and phosphocellulose columns to be analyzed by the activity blotting method. The protein elution pattern from the phosphocellulose column is shown in Fig. 3. The fractions A, B and C were collected and the proteins were concentrated by ultra-filtration. The concentrated fractions A, B and C, and the *E. coli* extract and the partially purified mouse APEX nuclease were fractionated by SDS-PAGE. Activity blotting on bleomycin-damaged DNA-fixed and acid-depurinated DNA-fixed membranes was conducted by capillary transfer, and the primed sites were detected by the non-radioactive procedure. The results of the activity blotting

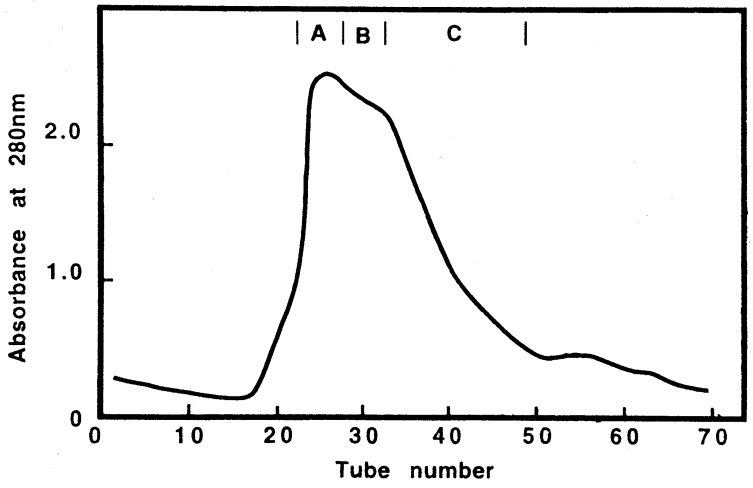

Fig. 3. Phosphocellulose chromatography of salt extract of human liver. Human liver APEX nuclease was extracted and partially purified by sequential chromatographies with DEAE-cellulose and phosphocellulose as described in MATERIALS AND METHODS. APEX nuclease adsorbed on a phosphocellulose column (10 × 330 mm) was eluted with 70 ml of 0.3 M potassium phosphate buffer. The elution was started at the position of tube No. 0. Each was 1 ml fraction. To use the eluant for the activity blotting, the main peak was divided into fractions A (tube No. 23 to 27), B (tube No. 28 to 32) and C (tube No. 33 to 47) as shown in the figure.

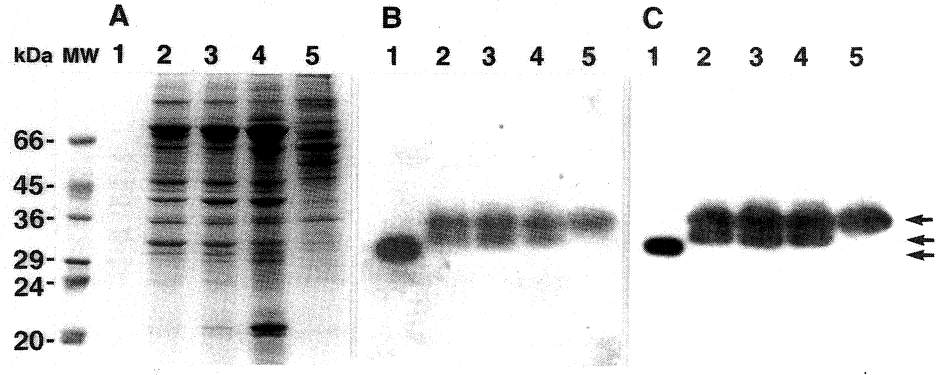

Fig. 4. Non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities of human liver APEX nuclease by the capillary blotting procedure of the activity blotting method. The sample and the method of the activity blotting were described in MATERIALS AND METHODS and the legend to Fig. 3. A, the CBB-stained gel. B, proteins of the renatured gel were capillary-blotted on bleomycin-damaged DNA-fixed membrane. C, proteins of the renatured gel were capillary-blotted on acid-depurinated DNA. The samples electrophoresed were the *E. coli* HB101 strain cell extract (0.5 μg protein) in lane 1, the concentrated fraction A (24 μg) in lane 2, fraction B (23 μg) in lane 3 and fraction C (39 μg) in lane 4, and the F_3 fraction of mouse APEX nuclease in lane 5. The positions indicated by the right upper, middle and lower arrows are the bands of 35 kDa peptide (APEX nuclease), 33 kDa peptide and exonuclease III, respectively.

indicate that the peptides electrophoresed at about 35 kDa and 33 kDa have priming activity for DNA polymerase on both bleomycin-damaged and acid-depurinated DNAs (Fig. 4). The 35 kDa peptide having the priming activity is thought to be human liver APEX nuclease itself. The 33 kDa peptide is thought to be the 33 kDa C-terminal peptide of APEX nuclease as has been reported previously.[8] The 33 kDa peptide of the B fraction of the human liver extract was directly compared with the known 33 kDa peptide fragment derived from the previously reported APEX hybrid protein[8] by activity blotting (Fig. 5). The results support the idea that the human liver 33 kDa peptide is the active 33 kDa C-terminal fragment of APEX nuclease. Previous results indicated that the 33 kDa C-terminal fragment was produced

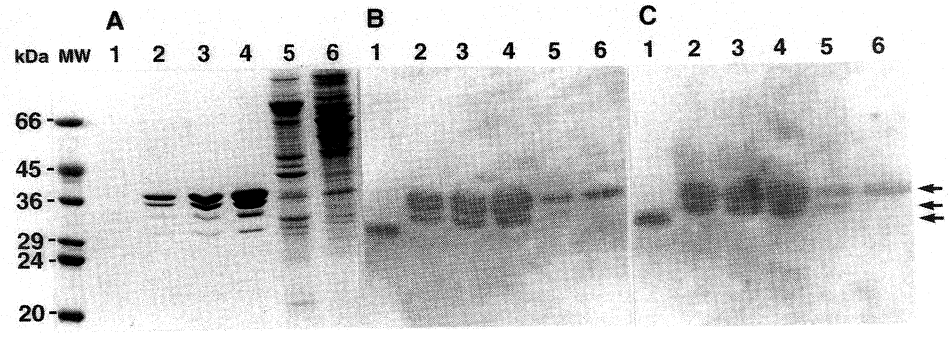

Fig. 5. Comparison between the 33 kDa C-terminal fragment derived from the purified APEX hybrid protein and the human liver 33 kDa peptide having APEX nuclease activity by non-radioactive, activity blotting method. The APEX hybrid protein and the enzymatically active C-terminal fragments were purified as described previously (8). A, the CBB-stained gel. B, proteins of the renatured gel were capillary-blotted on bleomycin-damaged DNA-fixed membrane. C, on acid-depurinated DNA. The samples electrophoresed were the *E. coli* HB101 strain cell extract (0.5 μg protein) in lane 1, the APEX hybrid protein fraction 15 μg in lane 2, 40 μg in lane 3 and 80 μg in lane 4, fraction B (16 μg) of human liver APEX nuclease in lane 5, and the F_3 fraction (35 μg) of mouse APEX nuclease in lane 6. The positions indicated by the right upper, middle and lower arrows are the bands of APEX nuclease, 33 kDa peptide and exonuclease III, respectively.

by proteolytic degradation of APEX nuclease during the extraction and purification of the enzyme. The Proteolytic enzyme involved in the degradation was suggested to be a mammalian subtilisin which belonging to dibasic processing endoproteases.[14,20] The 33 kDa peptide was observed sometimes in APEX nuclease fractions obtained from mouse ascites sarcoma cells and HeLa cells, and frequently when the process of the enzyme preparation took a long time.[14] The 33 kDa peptide seems to have appeared significantly in the present partially purified preparation of human liver APEX nuclease because of a postmortem change.

AP endonuclease activity and repair-initiating activity of partially purified human liver APEX nuclease

The activity blotting data indicate that the 35 kDa peptide of the human liver preparation has priming activities for DNA polymerase both on acid-depurinated DNA and on bleomycin-damaged DNA. This means that the peptide has 5′ AP endonuclease activity and DNA 3′ repair diesterase activity

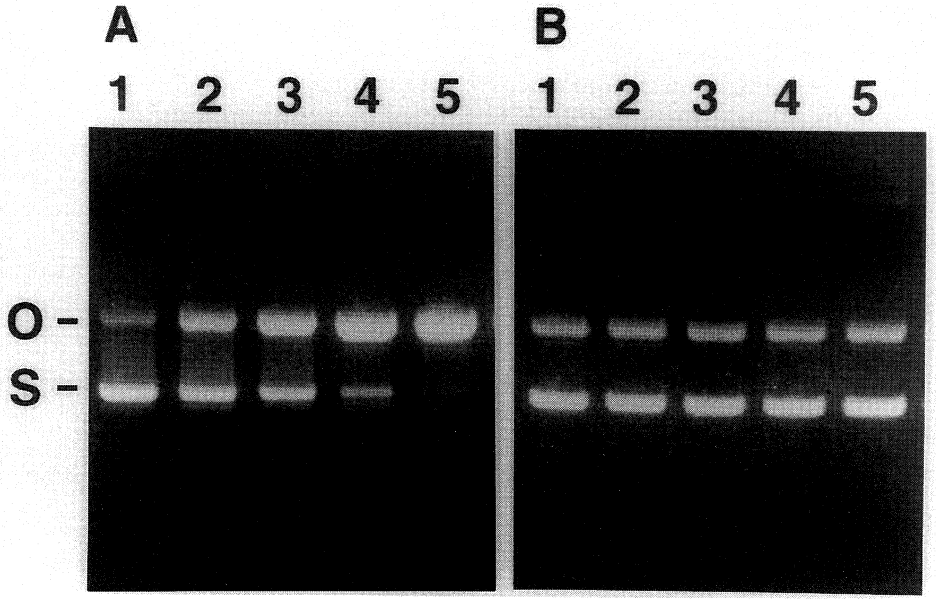

Fig. 6. Assay of AP endonuclease and non-specific endonuclease activities of partially purified human liver APEX nuclease. The fraction B shown in Fig. 3 was used as the partially purified APEX nuclease. Nicking activity of the fraction was measured as described previously.[12,21,22] Amount of DNA loaded was 0.4 μg per lane. A ; lane 1, acid-depurinated pUC18 DNA, lane 2, acid-depurinated pUC18 DNA incubated with the fraction B of 9×10^{-6} μg protein, lane 3, of 9×10^{-5} μg, lane 4, of 9×10^{-4} μg and lane 5, of 9×10^{-3} μg. B ; lane 1, native pUC18 DNA, lane 2, native pUC18 DNA incubated with the fraction B of 9×10^{-6} μg protein, lane 3, of 9×10^{-5} μg, lane 4, of 9×10^{-4} μg and lane 5, of 9×10^{-3} μg.

which can remove 3′ tags. The presence of the enzyme having 5′ AP endonuclease activity in the preparation was also confirmed by measuring the nicking activity of the preparation on acid-depurinated pUC18DNA (Fig. 6).

To analyze the priming activities of human liver APEX nuclease for repair of bleomycin-damaged and acid-depurinated DNAs, conformational analyses of damaged and repaired plasmid (pUC18) DNAs were perfomed as has been described previously.[21,22] The enzyme fraction supported repair of bleomycin-damaged DNA and acid-depurinated DNA in the presence of Klenow polymerase, 4 dNTPs, DNA ligase and ATP (Fig. 7). These results indicate again that the enzyme has 5′ AP endonuclease activity and DNA 3′ repair diesterase activity and is involved in repair of AP sites and single-strand breaks with 3′ tags. They also indicate that the 35 kDa peptide is actually APEX nuclease.

CONCLUSION

The author applied a non-radioactive activity blotting method for the detection of DNA repair enzymes to the detection of the human liver APEX nuclease. The method is useful for detecting and characterizing the enzyme even in crude preparations. The molecular mass of the repair enzyme can be estimated by this method, and a semiquantitative comparative assay of the

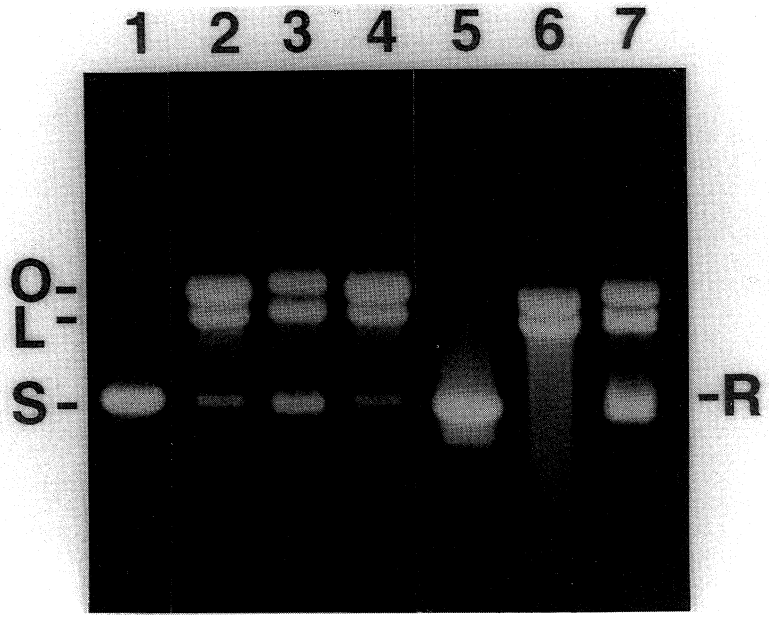

Fig. 7. Priming activity of partially purified human liver APEX nuclease for DNA repair of bleomycin-damaged and acid-depurinated pUC18 DNA. Conformational analysis of DNA repair using bleomycin-damaged and acid-depurinated DNAs was conducted as described previously.[21,22] Amount of DNA loaded was 0.25 μg per lane. Lane 1, control native DNA; lane 2, bleomycin-damaged DNA; lane 3, bleomycin-damaged DNA incubated at 37°C for 3 h for DNA repair with the complete assay mixture containing human liver APEX nuclease fraction (fraction B, 9 μg), 0.02 unit Klenow polymerase, 50 μM each of dNTPs, 40 unit T4 DNA ligase and 2.5 mM ATP; lane 4, bleomycin-damaged DNA incubated for DNA repair with the complete assay mixture omitted the APEX nuclease fraction; lane 5, acid-depurinated DNA; lane 6, acid-depurinated DNA incubated at 37°C for 3 h with the APEX nuclease fraction; lane 7, acid-depurinated DNA incubated at 37°C for 3 h for DNA repair with the complete assay mixture. Repaired DNAs in lanes 3 and 7 are electrophoresed at the position indicated by the symbol R.

enzyme is possible. An active fragment of the enzyme can be detected. This non-radioactive procedure is very convenient and safe as compared with the radioactive procedure. With this improvement this method, it may be used for the zymographical detection of many kinds of DNA-modifying (repair) enzymes and for the screening of disorders of DNA-modifying (repair) enzymes in clinical specimens.

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