Journal of Chromatography, 618 (1993) 147–166
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6818

Review

Activity gel and activity blotting methods for detecting DNA-modifying (repair) enzymes

Shuji Seki*, Kosuke Akiyama, Sekiko Watanabe and Ken Tsutsui

Department of Molecular Biology, Institute of Cellular and Molecular Biology, Okayama University Medical School, 2-5-1, Shikatacho, Okayama 700 (Japan)

(First received January 13th, 1993; revised manuscript received March 3rd, 1993)

ABSTRACT

Zymographical methods (activity gel, overlay gel, activity blot and activity blotting) for detecting DNA-modifying (repair) enzymes are reviewed. Emphasis is put on the novel activity blotting method in which DNA repair enzymes electrophoresed on a gel are blotted and detected on a damaged DNA-fixed nylon membrane. Its practical procedures, including a non-radioactive detection procedure, and representative results are also described.

CONTENTS

* Corresponding author.

0378-4347/93/$24.00

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CBB	Coomassie brilliant blue R-250
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetate
kDa	Kilodalton
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
SSC	Standard saline citrate consisting of 0.15 *M* NaCl and 0.015 *M* sodium citrate (pH 7.0)
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TEN buffer	A buffer consisting of 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA and 100 m*M* NaCl
Triton/buffer B	A solution consisting of 0.0175% Triton X-100, 0.25 *M* sucrose, 10 m*M* Tris–HCl, 4 m*M* $MgCl_2$, 1 m*M* EDTA and 6 m*M* 2-mercaptoethanol (pH 8.0, adjusted at 25°C)

1. INTRODUCTION

Contrary to the impression that cellular DNA is stable and genetic information is maintained invariably, a large amount of DNA damage can occur in cellular DNA. The constituents, such as bases, deoxyribose and phosphate, are constantly renovated by cellular repair mechanisms [1–3]. Sometimes these mechanisms fail, resulting in genetic changes or cell death. The genetic changes are possible causes of cellular transformation, ageing, molecular diseases and genetic diversity [1,4–7]. Various (several tens or more) types of DNA damage are produced in cells exposed to physical, chemical and biological stresses [1–7]. The lesions are generally repaired by cellular DNA repair systems to their original state [1–3]. To understand the mechanisms of genetic maintenance and their relation to genetic changes, knowledge of DNA repair systems is very important.

A typical DNA repair is known to occur in the following sequence: priming (recognition of DNA damage and incision–excision reaction); repair DNA synthesis; repair patch ligation; and chromatin reorganization. The complexity of DNA repair mechanisms is thought to be due mainly to diversity in the priming step. Several ten kinds of repair enzyme largely engaged in the priming step are thought to be present in mammalian cells, although at present only several enzymes that catalyse the priming reaction for specific types of DNA damage have been purified and characterized [1–7]. To clarify DNA metabolism (including repair and mutation), further studies on DNA-modifying (repair) enzymes are necessary.

Zymography (activity staining of enzymes sep-

arated by gel electrophoresis) of DNA-modifying enzymes is very useful for their molecular-mass estimation, characterization (or identification) and purification. Various zymographic methods for detecting DNA-modifying enzymes have been developed. The strategies for these methods are divided roughly into two categories. In one type of strategy, fractionation of proteins and detection of DNA-modifying enzymes are conducted in the same gel. This procedure, frequently referred to as "activity gel", uses DNA-embedded gels for protein separation and detection (reviewed in refs. 8 and 9). In the other type of strategy, fractionation of proteins is performed first in a standard gel, and then a substrate DNA-impregnated gel, paper or membrane is overlaid on the electrophoresed gel for detection of the target DNA-modifying protein (reviewed in ref. 9). Three different procedures (overlay gel, activity blot and activity blotting on damaged DNA-fixed membranes) are included in this activity transfer category.

General and extensive reviews on staining for enzymes (including DNA-modifying enzymes) in gels have been published [9–11]. Activity gel analysis of DNA-modifying enzymes has also been reviewed [8,9,12]. In the present paper, descriptions of the procedures for activity gels, overlay gels and activity blot are restricted to the essentials. Emphasis is laid on the novel activity blotting technique for detection of DNA repair enzymes [13–16], in which DNA repair enzymes electrophoresed on a gel are blotted on damaged DNA-fixed membranes and then the blotted membrane is processed for detection of the target DNA repair enzymes.

2. ACTIVITY GEL

2.1. Activity gel analysis of nuclease

In activity gel analysis for DNA-modifying enzymes, the substrate DNA (native, modified or synthetic) is immobilized in the acrylamide gel. Proteins are electrophoretically separated using the gel, and the activity of the target DNA-modifying enzyme is detected by an appropriate detection method on the gel. DNases electrophoresed in native disc polyacrylamide gels were first demonstrated on the gels in bands of hydrolysed DNA that did not stain with a DNA-binding dye, methyl green [17]. A sensitive fluorometric method for the detection of DNases on DNA-polyacrylamide gels was developed using closed circular-duplex DNA and ethidium bromide as a DNA-binding dye [18].

Protein separation in native gels containing DNA is frequently disturbed by charge interaction between DNA and protein. To overcome this difficulty, Rosenthal and Lacks [19,20] developed a system in which nucleases denatured by sodium dodecyl sulphate (SDS) were electrophoresed in SDS-polyacrylamide gels containing nucleic acid, and the nuclease activities were detected on the gels after renaturation of the enzymes by removal of SDS, as schematically shown in Fig. 1A. This system has many advantages, when the enzyme can be renatured after SDS polyacrylamide gel electrophoresis (PAGE) [19]. Because all proteins are rendered negatively charged on denaturation with SDS, all nucleases, regardless of isoelectric point, can be separated and detected. Molecular masses of proteins electrophoresed on the gels can be estimated by using appropriate protein-size markers, because protein separation by SDS-PAGE is based on molecular mass. Hydrophobic proteins as well as hydrophilic proteins can be analysed. Many modifications of these methods [19,20] and applications for various enzymes [21–28] have been reported.

2.2. Activity gel analysis of DNA polymerase and primase

To detect DNA polymerase by activity gels, a sample containing DNA polymerase is electrophoresed in a gel with immobilized, gapped (activated) calf thymus DNA. After the treatment of protein renaturation, the gel is incubated in a substrate mixture in which one of four deoxyribonucleoside triphosphates (dNTPs) is α-^{32}P-labelled. After the gel has been washed with 5% trichloroacetic acid (TCA) to remove unincorpo-

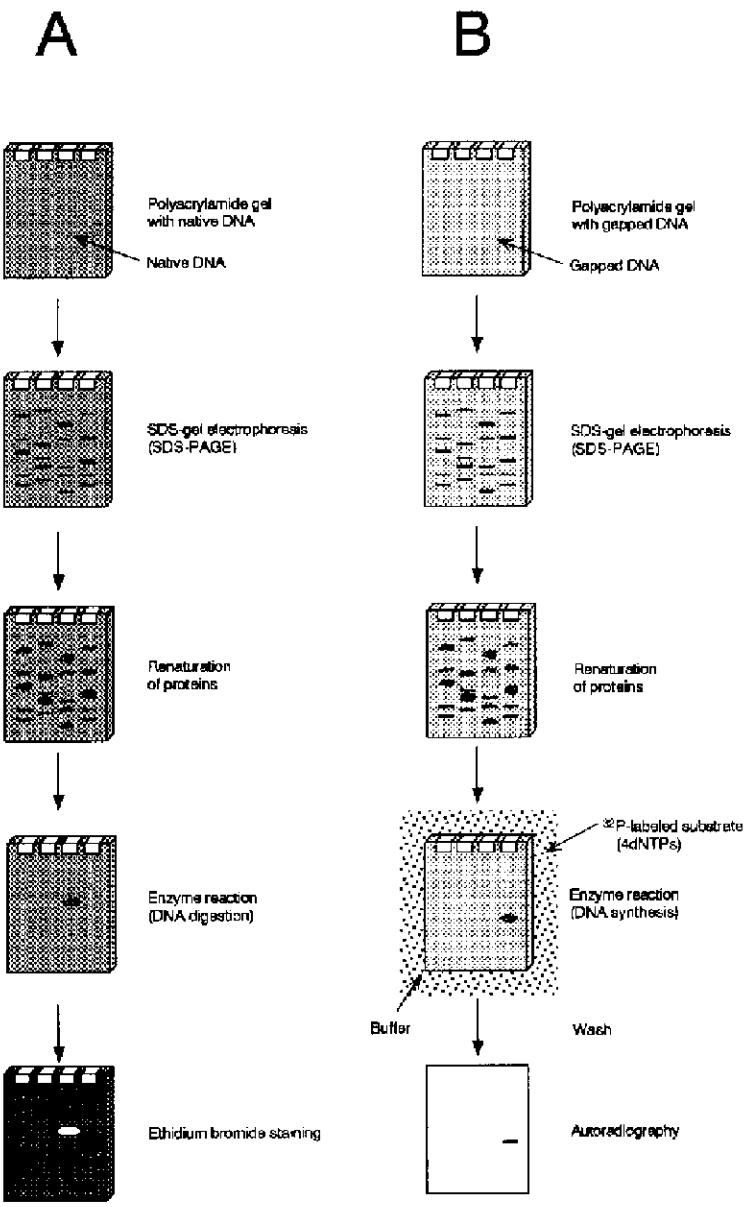

Fig. 1. Schematic representation of the activity gel methods for detecting endodeoxyribonuclease (A) and DNA polymerase (B).

rated radioactivity, and dried on a Whatman 3MM filter, the radioactivity incorporated into the DNA is detected at the vicinity of the polymerase band by autoradiography [29], as schematically shown in Fig. 1B. The gel can subsequently be stained with Coomassie brilliant blue (CBB) to determine the molecular mass of the enzyme involved. The present system is also used to detect exonuclease (5′-3′ and 3′-5′) by using ^{32}P-labelled gapped DNA in place of the unlabelled DNA [29]. Blank and co-workers [30,31] reported a modified procedure for promoting the renaturation of enzymes, in which fibrinogen was embedded in gels, and detergent was washed out from gels with aqueous isopropyl alcohol after electrophoresis. The method for detecting activ-

ity of DNA polymerase after electrophoresis of denatured proteins in DNA-SDS-polyacrylamide gels was termed the "activity gel" method [32]; at present, this term is used generally to indicate the method for detecting enzymes with nucleic acid-modifying activities in nucleic acid-containing gels after gel electrophoresis. Primase associated with DNA polymerase-α is also detected using a gel containing unprimed, single-stranded M13 DNA [33].

Modifications of these methods and applications for detecting DNA polymerases [31,34–40], active subunits of DNA polymerase-α [32,33, 41–45], DNA polymerase-β [46,47], DNA polymerase-γ [48] and DNA polymerase-δ [49] have been reported.

Longley and co-workers [50–52] have recently reported simultaneous *in situ* detection of DNA polymerase and associated exonuclease following SDS-PAGE. In this method, purified DNA polymerases are separated by electrophoresis through SDS polyacrylamide gels containing ^{32}P-labelled synthetic oligodeoxyribonucleotide primers annealed to M13mp2 DNA templates. Following electrophoresis and SDS removal, the enzymes are allowed to renature and express catalytic activities. Oligodeoxyribonucleotides modified by the catalytic activities are resolved from the gel by a second dimension of electrophoresis through a denaturing DNA sequencing gel. By using modified [^{32}P]oligonucleotides annealed to M13 DNA, *in situ* activities of exonuclease, DNA glycosylase, endonuclease and DNA ligase are also detected by this method [51,52].

2.3. Activity gel analysis of poly(ADP-ribose) polymerase

Poly(ADP-ribose) polymerase catalytic activity is detected by activity gel techniques essentially on the same principle as for nucleases and DNA polymerases. Poly(ADP-ribose) polymerase is electrophoresed on SDS polyacrylamide gels containing activated DNA. After renaturation of the enzyme, the gels are incubated with [^{32}P]NAD^+ for assay of poly(ADP-ribose) polymerase activity. Non-incorporated [^{32}P]NAD^+ is removed by washing with 5% TCA solution. Auto-[^{32}P]ADP-ribosylated enzymes in polyacrylamide gels are detected by autoradiography [47,53–55].

2.4. Activity gel analysis of DNA ligase

DNA ligase activity is detected after electrophoresis on SDS-polyacrylamide gels containing a poly(dA):5′-^{32}P-oligo(dT) substrate [56–59]. After electrophoresis, the proteins within the gel are renatured by washing, and the gel is incubated with 1 m*M* ATP and 5 m*M* $MgCl_2$ for *in situ* DNA ligase reaction. The gel is then washed and treated with calf intestinal alkaline phosphatase to remove 5′-^{32}P-ends of the unligated oligo(dT). DNA ligase activity on the gel is demonstrated by autoradiography [56–59].

2.5. Activity gel analysis of other DNA-modifying enzymes

DNA methyltransferase is electrophoresed on DNA-SDS-polyacrylamide gels and renatured. Its activity is assayed by incubating the gel with [methyl-^{3}H]S-adenosylmethionine and detected by fluorography [60,61]. DNA 3′ repair diesterase [62,63], terminal deoxynucleotidyl transferase [64], polynucleotide phosphorylases [65–67], ribonuclease H1 [68] and reverse transcriptases [69–71] are also analysed by the activity gel method.

3. OVERLAY GEL

Protein separation in native gels containing DNA is frequently disturbed by charge interaction between DNA and protein. Overlay gel methods have been developed to avoid the disturbance and to detect activities of DNA-modifying enzymes after PAGE under non-denaturing conditions [38,72–78]. In the representative method, proteins are separated in a polyacrylamide gel without DNA under non-denaturing conditions, and then an overlayer, made from agarose gel containing DNA and ethidium bromide, is placed on top of the polyacrylamide gel [73].

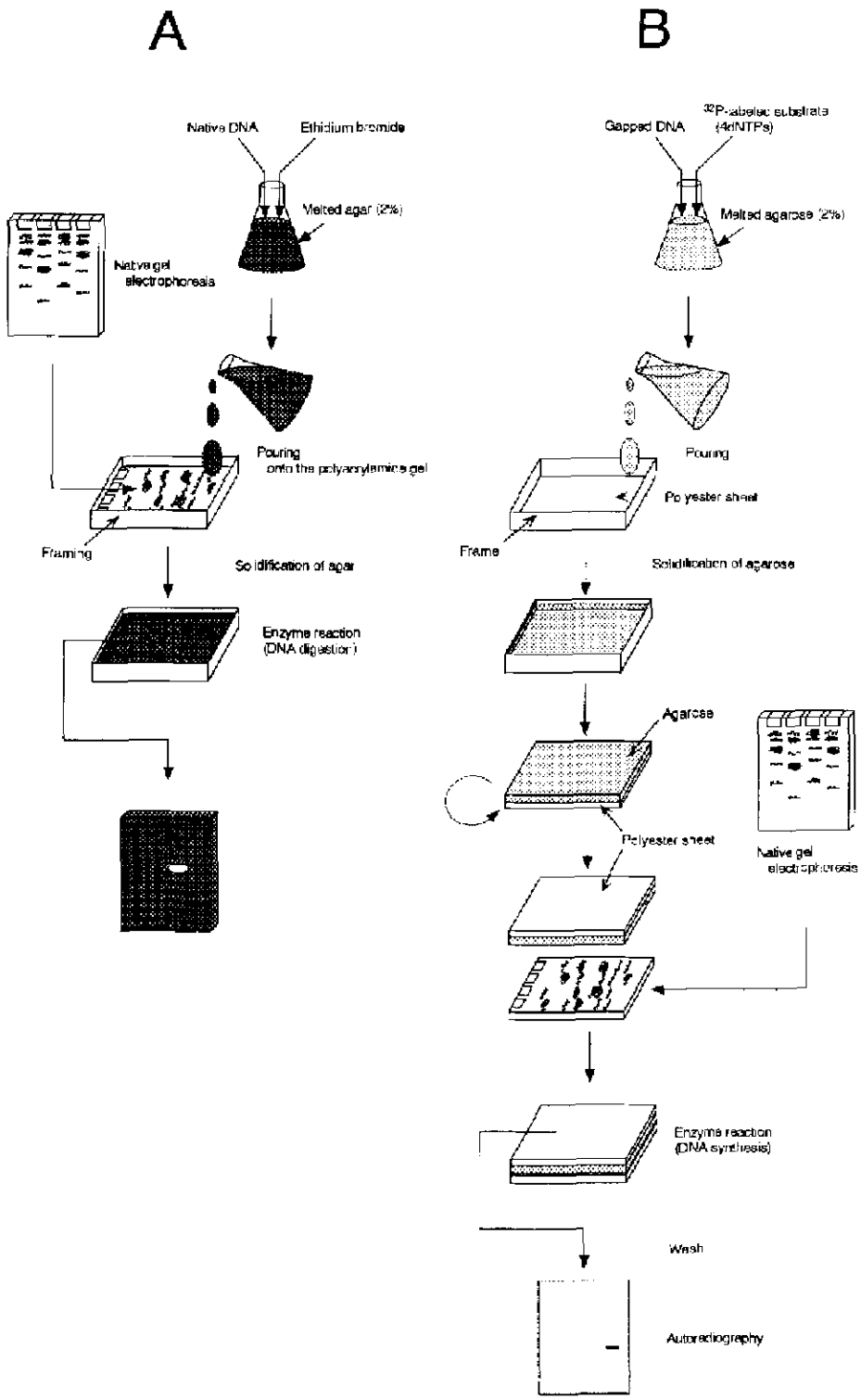

Fig. 2. Schematic representation of the overlay gel methods for detecting endodeoxyribonuclease (A) and DNA polymerase (B).

DNase activity is detected as a dark band on a fluorescent background on the overlay gel, as schematically shown in Fig. 2A. Yasuda *et al.* [77] devised a thin agarose film containing DNA and ethidium bromide for the overlayer and got good results for the analysis of DNase isozymes. Besides DNase, DNA polymerases separated in native polyacrylamide gels are also demonstrated using overlay gels containing activated DNA and four dNTPs, one of which is a ^{32}P-labelled nucleotide (Fig. 2B) [38,74–76]. Overlay gel methods are thought to have several advantages: (1) oligomeric enzymes can be detected; (2) monomeric enzymes, which are difficult to renature, may be detected; (3) protein separation based on their isoelectric points can be used to identify enzymes, as reported previously [73,77].

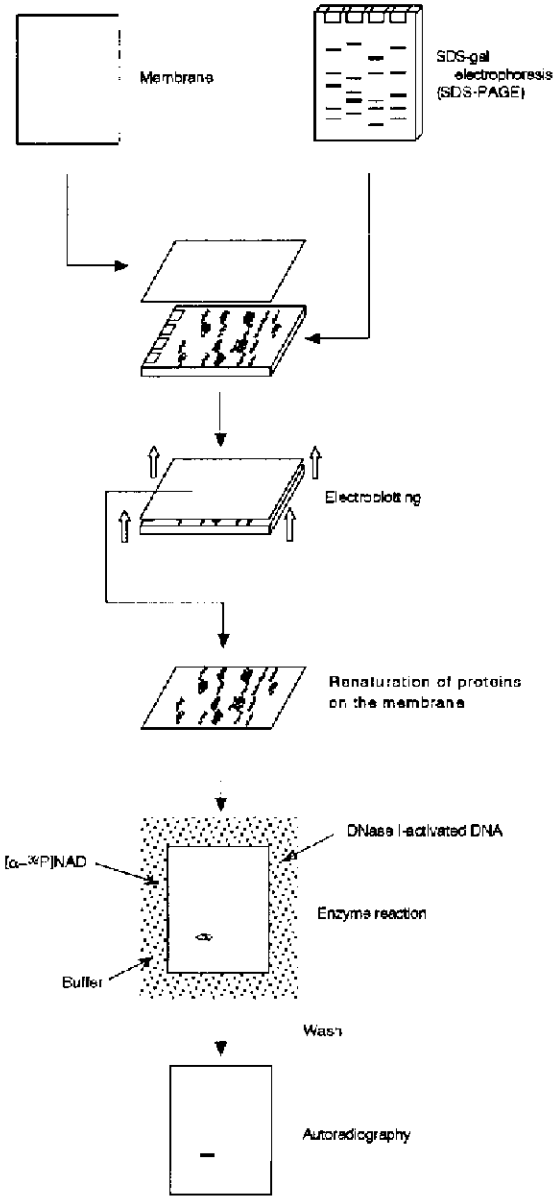

Fig. 3. Schematic representation of the activity blot method for detecting poly(ADP-ribose) polymerase.

4. ACTIVITY BLOT

The activity blot method was devised to detect poly(ADP-ribose) polymerase electrophoresed on polyacrylamide gels, as schematically shown in Fig. 3 [79]. Calf thymus poly(ADP-ribose) polymerase or a crude extract containing the enzyme is separated on SDS-polyacrylamide gels. The gels are incubated in the presence of 2-mercaptoethanol. Separated proteins are blotted on a nitrocellulose sheet. The blotted sheet is incubated in a renaturation buffer containing 0.3% (v/v) Tween 20, DNase I-activated DNA and divalent cations (Zn^{2+} and Mg^{2+}). The blotted membrane is incubated in the renaturation buffer supplemented with [adenylate-^{32}P]NAD, and then washed with the renaturation buffer. The blots are dried and analysed by autoradiography [79]. The method is applied to achieve direct detection of bacterial colonies transformed with the human poly(ADP-ribose) polymerase expression plasmid [79].

McLaren *et al.* [80] described a similar method for isolating Chinese hamster ovary (CHO) cells deficient in poly(ADP-ribose) polymerase activity by direct screening of colonies replica-plated onto nylon cloth.

5. ACTIVITY BLOTTING

The activity blotting method has been devised to detect DNA repair enzymes electrophoresed on acrylamide gels [13–16]. The method consists of the following six steps: (1) preparation of crude, partially purified or purified enzymes; (2) SDS- (denatured) or native (non-denatured)

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) detection of the activity-blotted site(s) to localize the repair enzyme.

The incision and/or excision provide priming sites for repair DNA synthesis. The blotted membrane is incubated with DNA polymerase in the presence of α-^{32}P-labelled or non-radioactively (digoxygenin-) labelled substrate. The site of substrate incorporation on the membrane that reflects the molecular mass of the repair (priming) enzyme is finally visualized by autoradiography or fluorescent staining of digoxygenin. Each step can be modified for adaptation to the target enzyme. The activity blotting method for the detection of enzymes involved in the priming step of DNA repair is schematically illustrated in Fig. 4.

Steps 1–3 described above are common to the methods of the activity gel, overlay gel, activity blot and activity blotting. Among these steps, the renaturation step is particularly important and has been discussed extensively in previous papers which we refer later. Steps 4–6 are unique to the activity blotting method, and are also referred to in the following sections.

5.1. Preparations of cell extracts and partially purified DNA repair enzymes

There is no special enzyme preparation procedure for the zymography of nucleic acid-modifying enzymes. An appropriate preparation procedure for the target enzyme should be selected from previously reported papers or newly devised. We describe here preparation procedures for mammalian and *E. coli* major apurinic/apyrimidinic (AP) endonucleases used for demonstration of the activity blotting technique.

Mammalian major AP endonuclease (designated as APEX nuclease) was extracted from permeabilized mouse ascites sarcoma or HeLa cells with 0.2 *M* potassium phosphate buffer (pH 7.5) [14,81,82]. After adjustment of the potassium phosphate concentration to 0.1 *M*, the extract (N_1 fraction) was mixed with packed phosphocellulose equilibrated with 0.1 *M* potassium phosphate buffer and rocked gently at 4°C overnight. The phosphocellulose was washed with 0.1 *M* potassium phosphate buffer and then transferred to a column. The enzymes were eluted with 0.3 *M* potassium phosphate buffer. The eluent was diluted with deionized water to 0.1 *M* potassium phosphate or dialysed against 50 m*M* Tris–HCl and 1 m*M* EDTA (pH 8.0) to reduce the salt concentration, because electrophoresis of proteins on SDS-polyacrylamide gels was disturbed when the ionic strength of the sample buffer was high (the electrophoresed lane becomes wide). The phosphocellulose fraction (fraction N_2) concentrated by ultrafiltration was mixed with a one fourth volume of the four-fold-concentrated gel loading buffer for SDS-PAGE (see Section 5.2) [81,83]. The mixture, in an Eppendorf tube, was immersed in a bath of boiling water for 2 min and, after chilling, an aliquot of the mixture was applied to SDS-PAGE.

E. coli HB101 strain cells were suspended in 1 ml of the loading buffer for SDS-PAGE [83]. 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 applying on SDS-polyacrylamide gels.

5.2. SDS- (denatured) and native (non-denatured) polyacrylamide gel electrophoresis

Generally, Laemmli's buffer system [83] has been used for SDS-PAGE of the activity gel and activity blot methods. We also have used the buffer system with minor modifications and an 8 × 8.5 × 0.1 cm slab gel 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 persulphate. The separation

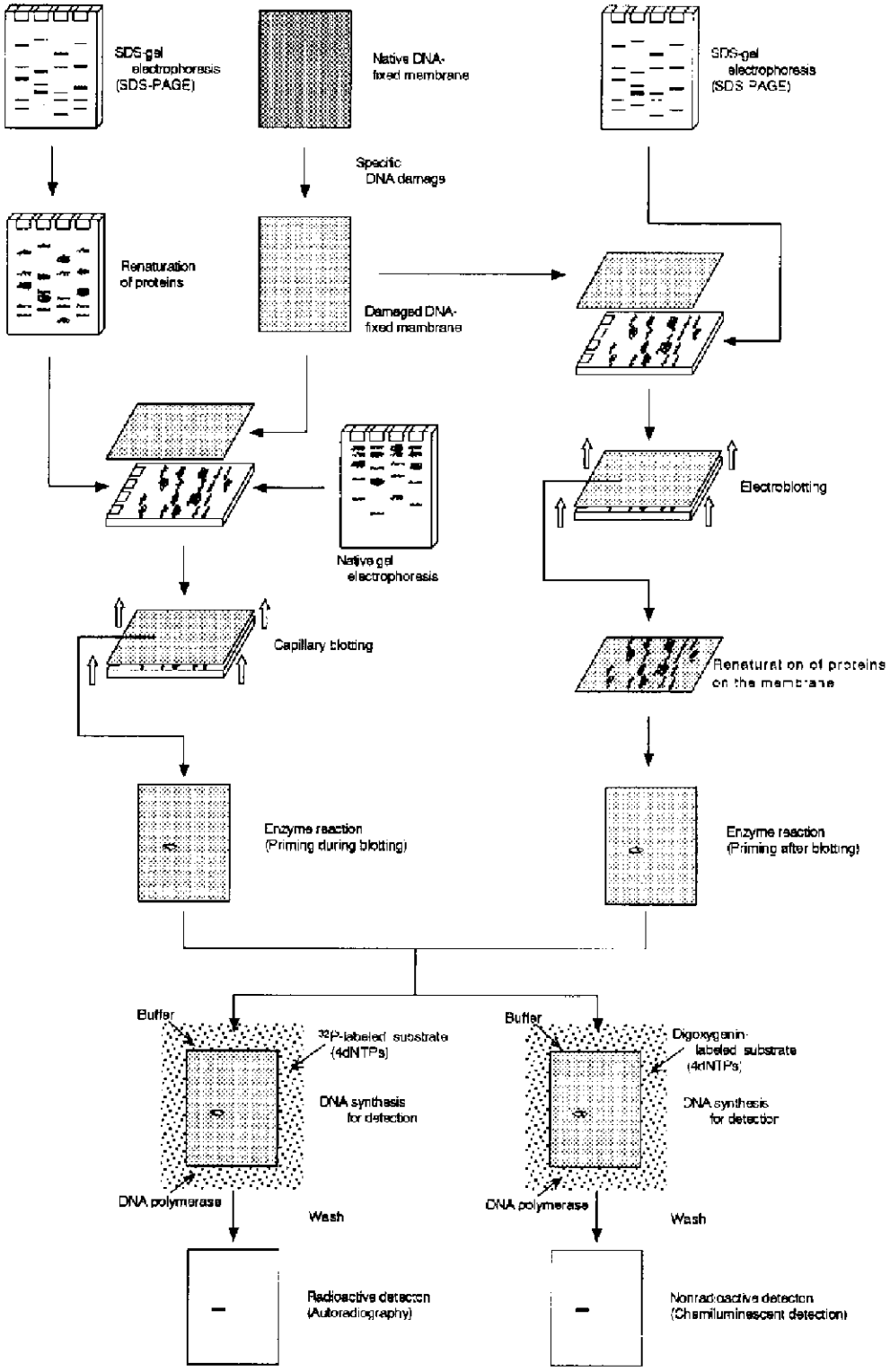

Fig. 4. Schematic representation of the activity blotting method for detecting priming enzymes for DNA polymerase on damaged DNA-fixed membranes.

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 persulphate. 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% bromophenol 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 (*ca.* 2–3 h).

Molecular mass markers used for SDS gel electrophoresis are MW-SDS-70L (Sigma, USA). Bovine serum albumin (BSA), which is added sometimes to the sample at a final concentration of 1 mg/ml and included in the gel at a concentration of 10 μg/ml to enhance the renaturation of the target enzymes and to stabilize the renatured enzymes in activity gels [12,13], is *not* added in the revised activity blotting technique, to prevent the contamination of nuclease and some other protein with BSA preparations [12].

Native (or non-denatured) PAGE, in which proteins are separated according to both size and charge, can be used for the activity blotting analysis of oligomeric enzymes and monomeric enzymes, which cannot renature fully after SDS denaturation (Fig. 4) [10], although the resolution is poor. Application of isoelectric focusing for the activity blotting method may increase the resolution [73,77].

5.3. Renaturation of enzymes in SDS-polyacrylamide gel

Electrophoresis carried out under denaturing and reducing conditions requires renaturation of the enzymes for activity detection. Lacks and Springhorn [84] showed that a number of enzymes are renaturable after SDS-PAGE. Enzymes appear to recover activity as soon as the SDS diffused out of the gel. Most monomeric enzymes can be renatured even after disruption of their disulphide bonds [84]. Oligomeric enzymes composed of identical subunits are poorly renaturable [84]. To remove SDS after SDS-PAGE, the gels are rinsed a few times in 50 m*M* Tris–HCl buffer with or without 1–2 m*M* EDTA (pH 7.5) and, if required, in the presence of 3–5 m*M* 2-mercaptoethanol [12,29]. Prolonged incubation of gels in 3–5 m*M* 2-mercaptoethanol is not recommended because softening of the gels is observed [29].

It has been shown that the RNase and DNase activities detected after SDS-PAGE vary widely, depending on the particular SDS preparation used for electrophoresis [30,31,85]. Blank *et al.* [31] suggested that lipophilic contaminants in some SDS preparations inhibited renaturation of DNA polymerase (also possibly other enzymes). To overcome the SDS variability by promoting enzyme renaturation, they reported a modification of the activity gel method in which fibrinogen was embedded in gels and, after electrophoresis, the gels were washed to remove the detergent with aqueous isopropyl alcohol [31]. In a study of catalytic peptides of mouse DNA polymerase using the activity gel method, Karawya and co-workers [32,34] found that the sensitivity for detecting purified enzymes was markedly increased by the addition of a heterogeneous mixture of proteins (a heat-inactivated crude homogenate of mouse myeloma or heat-treated fetal calf serum) to the enzyme sample prior to electrophoresis, and that it was essential to survey different lots of SDS to identify those that produce a high enzyme activity signal after renaturation.

Hager and Burgess [86] renatured the protein eluted from SDS-polyacrylamide gels as follows. The protein eluted was concentrated, and SDS was removed by acetone precipitation of the sample. Renaturation of the protein occurred after the precipitate was dissolved in 6 *M* guanidine hydrochloride and then diluted. According to the procedure, treatment with 6 *M* guanidine hydrochloride (a denaturation agent) and buffer wash of gels were used to renature calf thymus terminal deoxynucleotidyl transferase *in situ* after SDS-PAGE [64]. Fraser and co-workers [23,24] showed that many SDS preparations could be used for activity gel analysis when 0.1% Triton X-100 was included in the washing steps to remove SDS and the lipophilic contaminants. Si-

monin *et al.* [79] used a renaturation buffer containing 0.3% (v/v) Tween 20 in their activity blot analysis.

Necessary cofactors for enzyme renaturation and/or activity, such as divalent cations and/or reducing agents, should be added during the renaturation and/or reaction processes.

In the present activity blotting technique, the gel electrophoresed was rinsed briefly in a renaturation buffer consisting of 40 m*M* Tris–HCl (pH 8.0), 2 m*M* $MgCl_2$, 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 four changes of the buffer. The gel was left overnight at 4°C in fresh buffer with gentle shaking.

5.4. Preparation of damaged DNA-fixed membranes

This is a step unique to activity blotting analysis. Highly polymerized (calf thymus) DNA or closed circular (plasmid) DNA should be used for the preparation of native DNA-fixed membranes to minimize the background level or priming sites for DNA polymerase of the control, undamaged DNA. The stock solution of DNA (2.5 mg/ml in deionized water) is diluted with two-fold-concentrated standard saline citrate (SSC) to a final concentration of 0.145 mg/ml. A nylon membrane (Amersham Hybond-N, Boehringer positively charged nylon membrane or equivalent) (6 × 9 cm) prewetted in two-fold-concentrated SSC, is immersed in the DNA solution in a plastic beaker or in a scalable plastic bag, and incubated with gentle movement of the solution at room temperature for 1 h. After the incubation, the membrane is rinsed three times in two-fold-concentrated SSC, blotted on Kimwipe or a coarse filter paper to remove excess fluid, and then air-dried overnight in the dark. The DNA-fixed membrane is stored in a dark box until use. To make a damaged DNA-fixed membrane, the native DNA-fixed membrane is either treated with a solution of a DNA-damaging reagent, such as bleomycin, neocarzinostatin (a glycopeptide antitumour drug) or alkylating agents and DNase II, or irradiated with X-ray or ultraviolet light.

For example, the bleomycin-damaged DNA-fixed membrane is 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 m*M* Tris–HCl, 4 m*M* $MgCl_2$, 1 m*M* EDTA and 6 m*M* 2-mercaptoethanol, pH 8.0) supplemented with 5 μg/ml bleomycin A_2 and 30 μ*M* ferrous ammonium sulphate [13,87]. The neocarzinostatin-treated membrane is prepared by incubating the native DNA-fixed membrane at 37°C for 1 h in the dark with Triton buffer B supplemented with neocarzinostatin at 50 U/ml. To prepare an acid-depurinated DNA-fixed membrane, the native DNA-fixed membrane is incubated in 37.5 m*M* sodium citrate (pH 3.5) at 60°C for 30 min [13,87]. The acid depurination of DNA is conducted just before activity blotting to avoid spontaneous nicking. To prepare a DNase II-treated membrane, the native DNA-fixed membrane is treated at 37°C for 60 min with a solution consisting of 0.1 U of DNase II, 50 m*M* sodium acetate (pH 5.0) and 0.1% Triton X-100 [28]. After the treatment with DNA-damaging agents, the membranes are rinsed three times in two-fold-concentrated SSC, rinsed three times in a blotting buffer (40 m*M* Tris–HCl, pH 8.0, 2 m*M* $MgCl_2$, 0.02% sodium azide and 6 m*M* 2-mercaptoethanol), and used for protein blotting.

If the DNA damage can be induced in a DNA solution by a DNA-damaging agent, and the damaged structure is stable in a solution and during the preparation process of DNA-fixed membrane, we can use the damaged DNA to prepare a damaged DNA-fixed membrane. For example, the stock DNA solution is irradiated at ambient temperature, using a Toshiba Model KXC-18 X-ray unit at 100 Gy, and stored at −20°C until use [88]. The X-irradiated DNA is fixed on nylon membranes as described above. The same modification can be applied to prepare DNA-fixed membranes treated with bleomycin, neocarzinostatin, cisplatin, DNase I, DNase II or ultraviolet light.

The damaged DNA-fixed membranes can be prepared in advance and stored at room temperature in the dark until use, when the damaged DNA structure produced by a DNA-damaging agent is stable.

5.5. Protein (activity) blotting to damaged DNA-fixed membranes

The blotting of enzyme activity from gels on damaged DNA-fixed membranes can be done by the methods of capillary blotting, vacuum blotting and electroblotting.

5.5.1. Capillary blotting and vacuum blotting

The gel left overnight at 4°C in the fresh buffer (as described in the Section 5.3) is transferred to the blotting buffer described above (Section 5.4), and shaken gently for 30 min and for an additional 30 min in the fresh buffer.

Capillary transfer of enzyme activity from the renatured or native polyacrylamide gel to a damaged DNA-fixed nylon membrane is performed essentially as described for DNA transfer by Southern [89,90] with the following modifications. The renatured gel is placed on a transfer support covered with Whatman 3MM paper. The damaged DNA-fixed membrane is then placed on the gel, and proteins (activities) are transferred to the membrane with the blotting buffer (described in Section 5.4) at 30°C for 24–48 h. Necessary cofactors (such as divalent cations) for activity of the target enzyme should be included in the blotting buffer, because incision and/or excision (or DNA modification) by the enzymes occurs during the blotting process. After the blotting, the membrane is washed for 20 min with three changes of TEN buffer [10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA and 100 m*M* NaCl].

After the washing, the membrane can be proceeded directly to the next step (Section 5.6), or air-dried and stored for a while. The air-dried membrane is rinsed once in TEN buffer just before proceeding to the next step.

Fig. 5 shows an example of the results of radioactive detection after the capillary blotting of activities of human APEX nuclease, its 33-kDa C-terminal peptide and *E. coli* exonuclease III [15]. The bleomycin-damaged DNA-fixed membrane is used for the detection of DNA 3′ repair diesterase activity, which is involved in the conversion of 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, which catalyses incision of the 5′ side of AP sites to produce free 3′-hydroxyl termini. The resultant 3′-hydroxyl termini have priming activity for DNA polymerase which is included in the detection system of the present activity blotting. The result shown in

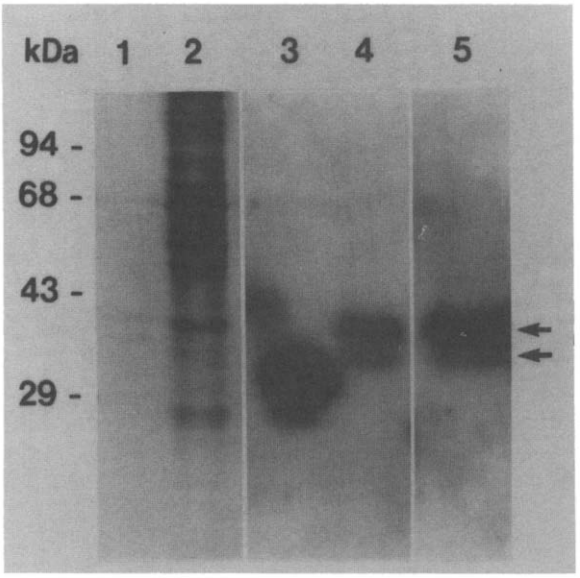

Fig. 5. Detection of priming activities for DNA polymerase of HeLa cell 35.4-kDa protein (APEX nuclease) and its 33-kDa peptide fragment on damaged DNA-fixed membranes by the activity blotting method. The samples were electrophoresed on SDS-polyacrylamide gels. Fractionated proteins were stained with CBB in lanes 1 and 2. Fractionated proteins were renatured and blotted by the capillary procedure on a bleomycin-treated DNA fixed membrane (lanes 3 and 4) or an acid-depurinated DNA-fixed membrane (lane 5). The membranes were incubated with Klenow polymerase in the presence of α-^{32}P-labelled substrate, and then the sites of substrate incorporation (primed sites) on the membranes were visualized by autoradiography. Lanes 1 and 3, the whole cell extract (1 μg protein per lane) of *E. coli* HB101 strain cells; lanes 2, 4 and 5, a preparation (fraction N_2 described in Section 5.1; 30 μg protein per lane) of APEX nuclease partially purified from HeLa cells. The major band detected in lane 3 and the upper and lower bands in lane 4 correspond to DNA 3′ repair diesterase activities of exonuclease III, APEX nuclease (indicated by the right upper arrow) and its 33-kDa peptide fragment (indicated by the right lower arrow), respectively. The upper and lower bands in lane 4 correspond to 5′ AP endonuclease activities of APEX nuclease and its 33 kDa peptide fragment, respectively.

Fig. 5 suggests that human APEX nuclease and its 33-kDa peptide fragment both show DNA 3′ repair diesterase and 5′ AP endonuclease activities, as reported previously [15]. The *E. coli* extract, which contains exonuclease III with DNA 3′ repair diesterase activity, is added to the detection system as an enzyme-positive control.

Vacuum transfer of the enzyme activity on damaged DNA-fixed membranes using vacuum blotting devices is based on the same principle as that for the capillary transfer [90].

5.5.2. *Electroblotting*

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to a damaged DNA-fixed membrane is performed by the western blotting technique [90–92] with the following modifications. After electrophoresis, the gels are preincubated at 37°C for 20 min in a reduction buffer containing 7 m*M* 2-mercaptoethanol, 192 m*M* glycine and 25 m*M* Tris–HCl (pH 8.3), with constant shaking. The incubation is repeated twice with the fresh reduction buffer. Electrotransfer of proteins onto a damaged DNA-fixed membrane is performed at 0°C and 70 V for 1 h. The electroblotted membrane is rinsed three times and soaked with gentle shaking at 4°C overnight in a renaturation buffer consisting of 40 m*M* Tris–HCl (pH 8.0 at 25°C), 2 m*M* $MgCl_2$, 0.02% sodium azide and 0.1% Triton X-100. The membrane is 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 is washed three times, for 20 min each time, with TEN buffer. After the washing, the membrane can be used directly for the next step (Section 5.6), or stored air-dried before the next step.

Fig. 6 shows an example of the result of non-radioactive detection of exonuclease III and APEX nuclease after the electroblotting of the *E. coli* extract and partially purified mouse APEX

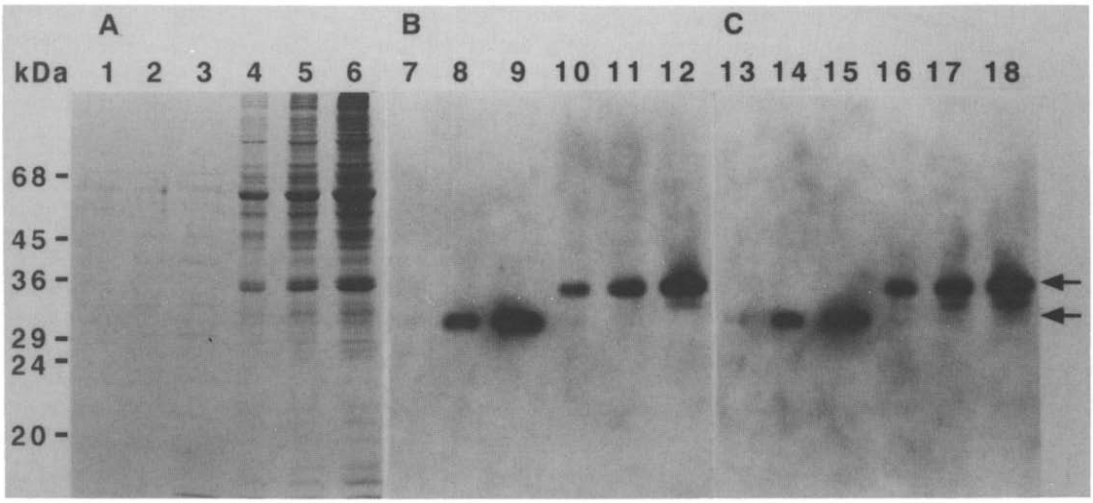

Fig. 6. Non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities by the electroblotting procedure of the activity blotting method. The *E. coli* HB101 strain cell extract (lanes 1–3) and the N_2 fraction (see Section 5.1) (lanes 4–6) of permeable mouse cell extract were electrophoresed on SDS-polyacrylamide gels. Native DNA was fixed on Hybond N (Amersham) and damaged as described in Section 5.4. Fractionated proteins were stained with CBB (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 membranes were incubated for DNA synthesis by the non-radioactive detection procedure (Section 5.6.2), and the sites of substrate incorporation (primed sites) on the membranes were visualized by the digoxygenin luminescent detection. The amount of the *E. coli* cell extract loaded was 1.8 μg in lane 1, 3.8 μg in lane 2, and 7.5 μg in lane 3. The amount of the N_2 fraction was 45 μg in lane 4, 90 μg in lane 5, and 180 μg in lane 6. Roughly dose-dependent positive signals are detected at the positions of 30 kDa (exonuclease III indicated by the right lower arrow) in lanes 1–3 and of 35.4 kDa (APEX nuclease indicated by the right upper arrow) in lanes 4–6. Positive signals at the 33-kDa position are due to the 33-kDa C-terminal peptide fragment of APEX nuclease.

nuclease electrophoresed on SDS-polyacrylamide gels. The bleomycin-damaged DNA-fixed and acid-depurinated DNA-fixed membranes are used for detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities, respectively. As reported previously [1,14,81], exonuclease III and APEX nuclease both have DNA 3′ repair diesterase and 5′ AP endonuclease activities, and are detected semi-dose-dependently.

5.6. Localization of DNA-modifying (repair) enzymes

The procedures for demonstrating the target enzyme on the activity-blotted membrane vary with the enzyme function and the damaged DNA fixed on the membrane. We have chosen DNA-modifying enzymes such as DNase I, *E. coli* exonuclease III and APEX nuclease, a mammalian major AP endonuclease, to illustrate the activity blotting. These enzymes have priming activity for DNA polymerase on appropriate DNAs by generating free 3′-hydroxyl termini.

The activity-blotted membrane is incubated with 3% BSA in TEN buffer at 37°C for 1 h to saturate the remaining protein binding sites, and then washed three times for 5 min each with a buffer [40 m*M* Tris–HCl (pH 8.0), 5 m*M* $MgCl_2$ and 50 m*M* NaCl]. The sites primed by the activity blotting are demonstrated by DNA synthesis in the presence of either radioactive substrate or non-radioactively labelled substrate.

5.6.1. Radioactive detection

To label the primed sites, the blotted, BSA-treated membrane is placed in a sealable plastic bag and incubated for DNA synthesis at 37°C for 30 min in 2.5 ml of a DNA polymerase substrate solution containing 40 m*M* Tris·HCl (pH 8.0 adjusted at 25°C), 5 m*M* $MgCl_2$, 50 m*M* NaCl, 5 m*M* 2-mercaptoethanol, 100 μM each dATP, dGTP and dTTP, 1 μM unlabelled dCTP, 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, Amersham), and 1 U of Klenow polymerase (or 2 U of DNA polymerase-β). A 2.5-ml volume of the reaction mixture is used for two sheets (attached back-to-back) of *ca.* 50 cm^2 each of the blotted membranes. After the incubation for DNA synthesis, the membrane is washed five times, for 10 min each time, with 5% (w/v) TCA to remove unincorporated nucleotides, blotted on paper towels and air-dried. The membrane is exposed at −60°C for 12–48 h to Fuji New RX X-ray film and DuPont Cornex Lightening-plus screens.

Many examples of activity blotting analysis using the radioactive detection procedure have been reported [13–16,82], and an example is shown in Fig. 5. Radioactive detection is rather more sensitive than non-radioactive, but the latter is more convenient than the former.

Some commercial preparations of ^{32}P-labelled nucleotides are contaminated with [^{32}P]polyphosphates. The contamination causes non-specific labelling of almost all protein bands blotted on damaged DNA-fixed membrane. We have had no such problems with the [^{32}P]dCTP from Amersham.

5.6.2. Non-radioactive detection

The blotted, BSA-treated membrane is placed in a sealable plastic bag and incubated for DNA synthesis at 37°C for 30 min in 2.5 ml of a DNA polymerase substrate solution containing 40 m*M* Tris–HCl (pH 8.0), 5 m*M* $MgCl_2$, 50 m*M* NaCl, 5 m*M* 2-mercaptoethanol, 100 μM each dATP, dGTP and dCTP, 65 μM dTTP, 35 μM digoxygenin-dUTP (Boehringer Mannheim) and 1 U of Klenow polymerase. After the DNA synthesis, the membrane is washed in 5% (w/v) TCA with three changes for 10 min each to remove unincorporated nucleotides, in two-fold-concentrated SSC with three changes for 10 min each, and in the same solution overnight.

Detection of the digoxygenin-labelled DNA is performed at room temperature using digoxygenin-luminescent detection kit (Boehringer Mannheim) according to the company protocol. Briefly, the membrane is washed shortly in buffer 1 (0.1 *M* maleic acid and 0.15 *M* NaCl, pH 7.5 adjusted with NaOH) supplemented with 0.3% (w/v) Tween 20, incubated for 30 min with 100 ml of 1% blocking reagent in buffer 1, and then incubated for 30 min in 20 ml of diluted anti-digoxygenin-alkaline phosphatase conjugate solution.

The membrane is washed twice, for 15 min each time, with buffer 1 supplemented with 0.3% Tween 20 to remover unbound antibody conjugate, equilibrated for 2 min with 20 ml of buffer 3 [0.1 *M* Tris–HCl (pH 9.5), 0.1 *M* NaCl and 50 m*M* $MgCl_2$] and incubated at room temperature for 5 min in *ca.* 10 ml of a diluted AMPPD [3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxyethane] solution. After removal of the solution and air bubbles from the sealable bag containing the membrane by gentle squeezing, and sealing of the bag, the membrane in the bag is incubated at 37°C for 15 min, and then exposed to an X-ray film at room temperature for 20–120 min, as for autoradiography. The blotted membrane may be used for immunological detection of the enzyme protein after the zymographic analysis.

Figs. 6 and 7 are both examples of the non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities, and Figs. 8 and 9 are examples of the non-radioactive detection of DNase I. Figs. 7 and 8 are examples of capillary blotting, whereas Figs. 6 and 9 are examples of electroblotting. Capillary blotting requires a longer processing time than electroblotting, but the detection sensitivities of the former are generally higher than those of the latter. The detection of the enzyme activities is dependent on the enzyme dose in both procedures, although the protein blotting is incomplete, especially in case of capillary blotting.

Other non-radioactive detection procedures, such as ECL (Amersham), may be applied to the activity blotting method.

5.7. DNA-modifying (repair) enzymes detected or possibly detected by the activity blotting method

The activity blotting method has been shown to be applicable to the detection of activities of endonucleases, such as DNase I, DNA 3′ repair diesterase, AP endonuclease, and DNA 3′ phosphatase (Figs. 5 9) [13–16,82]. Active peptide

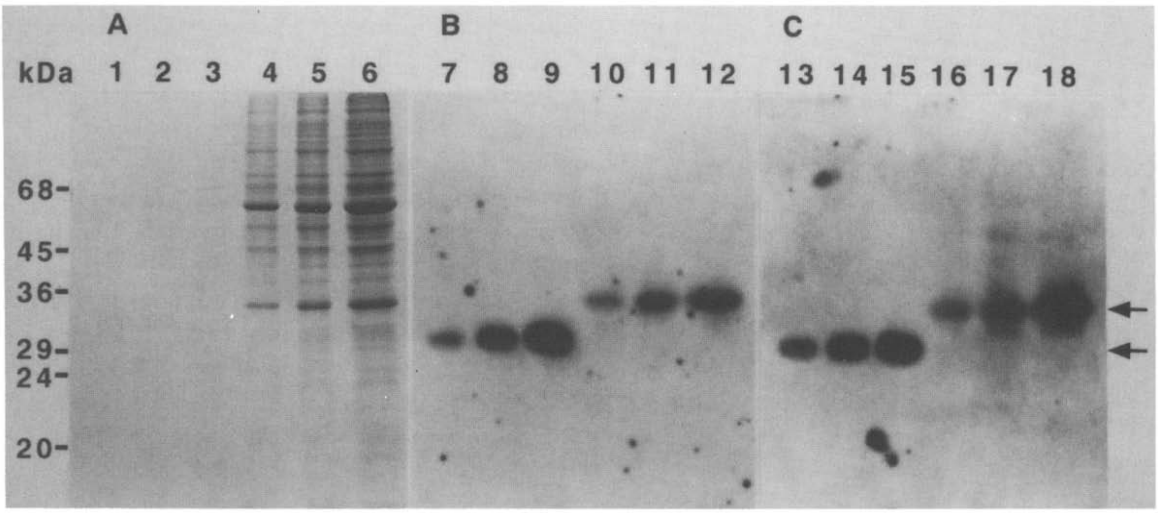

Fig. 7. Non-radioactive detection of DNA 3′ repair diesterase and 5′ AP endonuclease activities by the capillary blotting procedure of the activity blotting method. The sample and the method of the activity blotting were the same as described in Fig. 6, except that a positively charged nylon membrane (Boehringer Mannheim) was used in place of Hybond N, and the capillary blotting procedure was used in place of the electroblotting procedure. The amount of the *E. coli* cell extract loaded was 1.4 μg in lane 1, 2.8 μg in lane 2 and 5.6 μg in lane 3. The amount of the N_2 fraction was 45 μg in lane 4, 90 μg in lane 5 and 180 μg in lane 6. The positions indicated by the right upper and lower arrows are the bands of APEX nuclease and exonuclease III, respectively. The capillary blotting procedure gave almost the same result as the electroblotting procedure, although the enzyme-dose dependency of the signals is clearer in the former than in the latter.

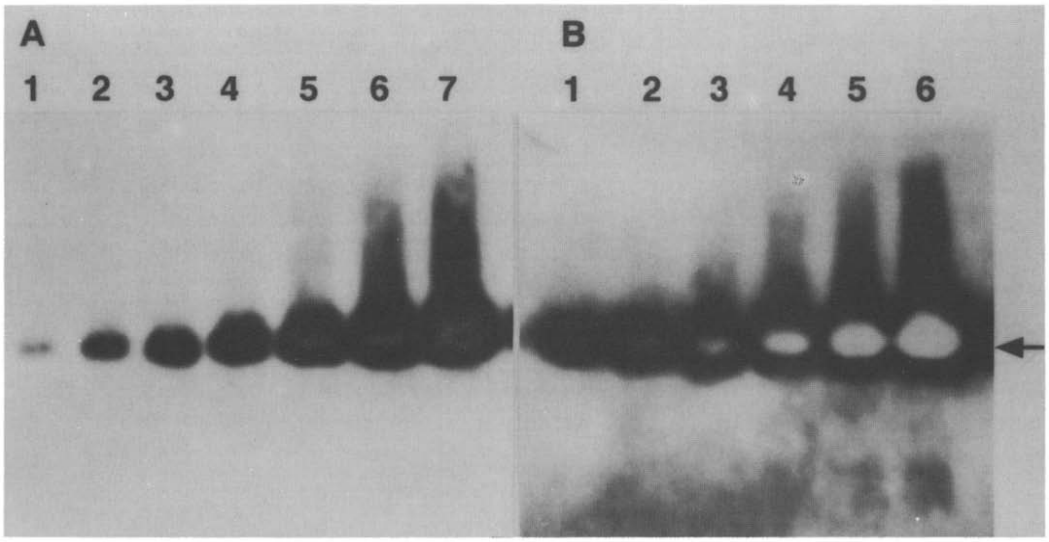

Fig. 8. Non-radioactive detection of DNase I by the capillary blotting procedure of the activity blotting method. DNase I (3.5 U/μg; 31 kDa) was obtained from Takara Shuzo (Kyoto, Japan). After SDS-PAGE of DNase I, the enzyme was renatured and blotted on a native DNA-fixed membrane. The membrane was incubated for DNA synthesis by the non radioactive detection procedure (Section 5.6.2), and the sites primed by DNase I and DNA-synthesized were detected by digoxygenin luminescent detection (indicated by an arrow). When a large amount of the enzyme was applied on a lane, a large positive signal with a clear centre is observed (B, lanes 3–6). The clear centre indicates a region in which DNA is largely digested. The amount (U) of DNase I loaded was: (A) 0.001 in lane 1, 0.0025 in lane 2, 0.005 in lane 3, 0.01 in lane 4, 0.025 in lane 5, 0.05 in lane 6 and 0.1 in lane 7; (B) 0.025 in lane 1, 0.05 in lane 2, 0.1 in lane 3, 0.25 in lane 4, 0.5 in lane 5 and 1.0 in lane 6. Experiments (A) and (B) were conducted separately.

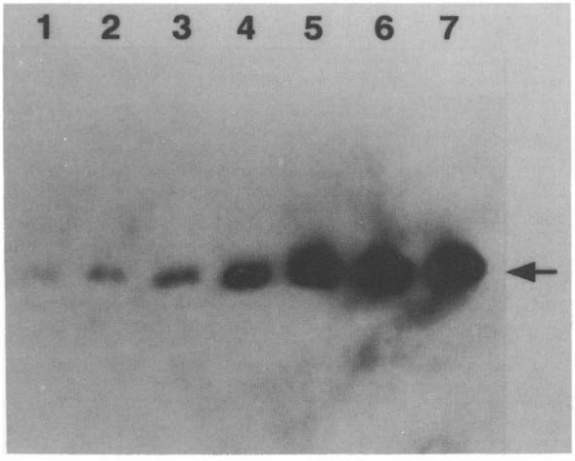

Fig. 9. Non-radioactive detection of DNase I by the electroblotting procedure of the activity blotting method. The sample and the method of the activity blotting were as described in Fig. 8, except that the electroblotting procedure was used in place of the capillary blotting procedure, and that the blotted membrane was washed twice with a solution containing 40 m*M* Tris–HCl (pH 8.0), 2 m*M* $MgCl_2$ and 6 m*M* 2-mercaptoethanol, and incubated at 30°C for 1 h in the same buffer prior to DNA synthesis. The amount (U) of DNase I loaded was 0.01 in lane 1, 0.025 in lane 2, 0.05 in lane 3, 0.1 in lane 4, 0.25 in lane 5, 0.5 in lane 6, and 1.0 in lane 7.

fragments derived from APEX nuclease and an active hybrid protein expressed in *E. coli* are also detected (Fig. 5) [13–16,82].

The method may be applied for detecting various other DNA-modifying enzymes by contriving the damaged DNA fixed on membranes and the detection system. For example, DNA ligase may be detected by blotting active DNA ligase in a gel on a poly(dA): 5'-^{32}P-oligo(dT)-fixed membrane, basically as described previously [13,56–59]. DNA polymerase and reverse transcriptase may be detected by using gapped DNA-fixed membranes and RNA-fixed membranes, respectively. Poly(ADP-ribose) polymerase and methyltransferase may be detected by electroblotting of protein on gapped DNA- and native DNA-fixed membranes, and incubating with [^{32}P]NAD^+ and [methyl-^{3}H]S-adenosyl methionine, respectively, by applying the previous procedures [53,60,79]. Various N-glycosylases may be detected by blotting N-glycosylase activity on a nylon-membrane-fixed DNA with appropriate base damage, such as O^7-methyladenine for O^7-

methyladenine DNA glycosylase and uracil for uracil N-glycosylase. The resultant AP sites on the membrane are detected by incubating the membrane with 5′-AP endonuclease (such as APEX nuclease), DNA polymerase and radiolabelled or non-radiolabelled substrate mixture for DNA synthesis. DNA binding protein for a modified DNA may be detected immunologically by blotting on the modified DNA-fixed membrane after gel electrophoresis.

6. ELUTION OF ENZYMES FROM GELS, RENATURATION AND PARTIAL PROTEIN SEQUENCING

The methods reviewed in this paper permit the identification of the band of an active DNA-modifying enzyme and its purification after gel electrophoresis (Fig. 10). When an enzyme is not characterized enough, further purification is necessary to characterize it. Zymography, combined with protein staining of the gel, provides valuable information on the enzyme purification. If chromatographic purification is not easy but zymographic analysis using SDS-PAGE is possible, it can be purified from the SDS-polyacrylamide gel of its partially purified preparations or from the electrophoresed gel of its crude preparations by a combination of isoelectric focusing and SDS-PAGE. The target enzyme can be purified by cutting out the enzyme band from the gel in which the band is well resolved from its surroundings. The enzyme protein is eluted from the gel band, renatured and characterized, basically according to the method of Hager and co-workers [86,93] and Ishii *et al.* [94].

Briefly, the protein band is visualized in the gel by light staining with CBB or KCl, cut out, and sliced into *ca.* 1-mm^3 pieces. The pieces are suspended in 2 *M* Tris–HCl buffer (pH 8.0) in an Eppendorf tube. The tube is rotated at room temperature for 15–60 min, then the sample is centrifuged, and the pellet is collected by removing the supernatant. The protein is eluted in an extraction buffer containing 50 m*M* Tris–HCl (pH 8.0), 0.1% SDS, 0.1 m*M* EDTA, 150 m*M* NaCl, 5 m*M* dithiothreitol (DTT) and 50 μg/ml BSA by rotating the tube at room temperature for 12–24 h, and the sample is centrifuged to collect the eluent. Protein in the eluent is precipitated at −80°C for 1 h or more with four volumes of cold acetone. SDS and CBB are removed in the acetone extract. The precipitate is washed with 80% ethanol–20% dilution buffer consisting of 50 m*M* Tris–HCl (pH 8.0), 0.1 m*M* EDTA, 150 m*M* NaCl, 1 m*M* dithiothreitol (DTT), 50 μg/ml BSA and 20% (w/v) glycerol. Ethanol and acetone are evaporated by centrifuging for 2–5 min in Speed-Vac concentrater (Sarvant). The precipitate is dissolved thoroughly in 20 μl of 6 *M* guanidine–HCl in the dilution buffer, and the solution is allowed to stand at 37°C for 1 h. The solution is then diluted 50-fold with the dilution buffer and permitted to renature for 12–24 h at room temperature. The enzyme activity of the sample is assayed directly or after concentration by ultrafiltration.

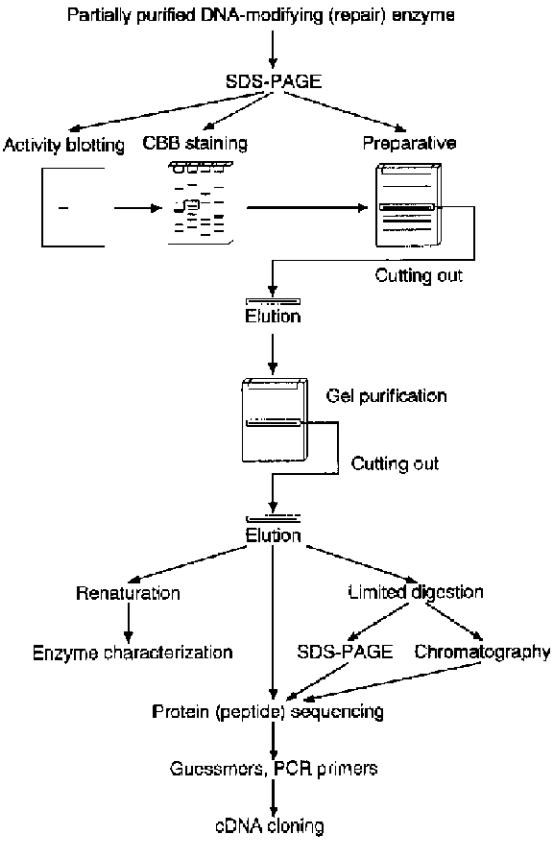

Fig. 10. Strategy for studying a DNA-modifying (repair) enzyme.

Determination of partial amino acid sequences of the target enzyme can be made possible by using the gel-purified preparations directly, or after partial digestion with protease. The band corresponding to the target enzyme or its peptide fragment on the CBB-stained polyacrylamide gel is excised, immersed and rotated in deionized water with several changes for one day to remove acetic acid. The gel pieces are crushed, immersed and rotated to extract the enzyme for one day in 50 m*M* ammonium bicarbonate solution (pH 10 adjusted with ammonium hydroxide) containing 0.1% SDS and dithiothreitol at 1 mg/ml, as described previously [95]. The extract is concentrated by ultrafiltration. The N-terminal amino acid sequence of the gel-purified protein is determined by a protein sequenator. By using the sequence information, cDNA cloning for the enzyme may become possible [14]. For further details on sequencing of the gel-purified protein, ref. 96 should be consulted.

7. CONCLUSION

Zymographic methods (activity staining of enzymes separated by gel electrophoresis) for detecting DNA-modifying (repair) enzymes have several advantages [8,9,11,12]: (1) enzymically active proteins can be detected and characterized even in crude preparations: in particular, their molecular masses can be estimated; (2) active precursors and degradation products of enzymes can be detected; (3) semiquantitative comparative assay of enzymes is possible: (4) enzymes obtained from cells in various stages, various tissue cells and various organisms can be compared; (5) isozymes, expressed enzymes, mutant enzymes and multifunctional enzymes can be identified; (6) zymography provides information for further purification of an enzyme, and a step in the determination of the primary structure of the enzyme.

Known zymographic methods for DNA-modifying enzymes can be divided into the activity gel and activity transfer methods. The latter methods include overlay gel, activity blot and activity blotting on damaged DNA-fixed membranes. Each method has both merits and demerits, and a selection should be made to suit the purpose. In the present review, emphasis is placed on a novel method of the activity blotting on damaged DNA-fixed membranes, because the other methods have been reviewed in detail previously [8,9,12]. The activity blotting method has wide applicability by selecting or contriving the damaged DNA-fixed membranes and detection methods. It is especially applicable to the detection of various DNA repair enzymes. The blotted membranes can be handled easily (washing, incubation, activity detection and so on), and non-radioactive detection is possible. By improving the method, it may be used for zymographic detection of many kinds of DNA-modifying enzyme, for detection of DNA binding proteins, for screening of bacterial colonies transformed with expression plasmid, and for screening of disorders of DNA-modifying enzymes in clinical specimens.

8. ACKNOWLEDGEMENTS

We are grateful to our colleagues Dr. K. Takatori and Mr. T. Nakamura for allowing us to cite unpublished data. We also thank Boehringer Mannheim Yamanouchi for providing the DIG luminescent detection kit, and Nippon Kayaku Co. for providing copper-free bleomycin A_2. This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Japan Ministry of Education, Science and Culture.

REFERENCES

1 E. C. Friedberg, *DNA repair*, W. H. Freeman and Company, New York, 1985.
2 T. Lindahl, *Annu. Rev. Biochem.*, 51 (1982) 61.
3 A. Sancar and G. B. Sancar, *Annu. Rev. Biochem.*, 57 (1988) 29.
4 L. H. Breimer, *Br. J. Cancer*, 57 (1988) 6.
5 L. A. Loeb and B. D. Preston, *Annu. Rev. Genet.*, 20 (1986) 201.
6 F. Giannelli, *J. Cell Sci., Suppl.* 4 (1986) 383.
7 S. I. S. Rattan, *Int. Rev. Cytol.*, 116 (1989) 47.
8 U. Bertazzoni, A. I. Scovassi, M. Mezzina, A. Sarasin, E. Franchi and R. Izzo, *Trends Genet.*, 2 (1986) 67.
9 D. M. Giersten and O. Gabriel, *Anal. Biochem.*, 203 (1992) 181.

10 O. Gabriel, *Methods Enzymol.*, 22 (1971) 578.
11 O. Gabriel and D. M. Gersten, *Anal. Biochem.*, 203 (1992) 1.
12 A. Spanos and U. Hübscher, *Methods Enzymol.*, 91 (1983) 263.
13 S. Ikeda, S. Seki, S. Watanabe, M. Hatsushika and K. Tsutsui, *Anal. Biochem.*, 192 (1991) 96.
14 S. Seki, K. Akiyama, S. Watanabe, M. Hatsushika, S. Ikeda and K. Tsutsui, *J. Biol. Chem.*, 266 (1991) 20797.
15 S. Seki, M. Hatsushika, S. Watanabe, K. Akiyama, K. Nagao and K. Tsutsui, *Biochim. Biophys. Acta*, 1131 (1992) 287.
16 K. Akiyama, S. Watanabe and S. Seki, *Abstracts of the 11th International Congress on Photobiology, Kyoto, Sept. 7–12, 1992*, Photobiology Association of Japan, Tokyo, 1992, p. 280.
17 J. B. Boyd and H. K. Mitchell, *Anal. Biochem.*, 13 (1965) 28.
18 D. J. Girdina, P. H. M. Lohman and R. R. Hewitt, *Anal. Biochem.*, 51 (1973) 255.
19 A. L. Rosenthal and S. A. Lacks, *Anal. Biochem.*, 80 (1977) 76.
20 S. A. Lacks, *J. Biol. Chem.*, 256 (1981) 2644.
21 T. Y.-K. Chow and M. J. Fraser, *J. Biol. Chem.*, 258 (1983) 12010.
22 S. A. Coughlin and D. M.-D. Green, *Anal. Biochem.*, 133 (1983) 322.
23 M. J. Fraser, T. Y.-K. Chow, H. Cohen and H. Koa, *Biochem. Cell Biol.*, 64 (1986) 106.
24 D. Ramotar, A. H. Auchincloss and M. J. Fraser, *J. Biol. Chem.*, 262 (1987) 425.
25 E. Käfer, A. Tittler and M. J. Fraser, *Biochem. Genet.*, 27 (1989) 153.
26 M. Mezzina, S. Nocentini, J. Nardelli, G. Renault, E. Moustacchi and A. Sarasin, *Mol. Carcinogenesis*, 2 (1989) 179.
27 M. P. Chang, R. L. Baldwin, C. Bruce and B. J. Wisnieski, *Science*, 246 (1989) 1165.
28 I. Harosh, D. M. Binninger, P. V. Harris, M. Mezzina and J. B. Boyd, *Eur. J. Biochem.*, 202 (1991) 479.
29 A. Spanos, S. G. Sedgwick, G. T. Yarranton, U. Hübscher and G. R. Banks, *Nucleic Acids Res.*, 9 (1981) 1825.
30 A. Blank, R. H. Sugiyama and C. A. Dekker, *Anal. Biochem.*, 120 (1982) 267.
31 A. Blank, J. R. Silber, M. P. Thelen and C. A. Dekker, *Anal. Biochem.*, 135 (1983) 423.
32 E. M. Karawya and S. H. Wilson, *J. Biol. Chem.*, 257 (1982) 13 129.
33 U. Hübscher, *EMBO J.*, 2 (1983) 133.
34 E. Karawya, J. A. Swack and S. H. Wilson, *Anal. Biochem.*, 135 (1983) 318.
35 P. Plevani, M. Foiani, P. Valsasnini and G. Badaracco, *J. Biol. Chem.*, 260 (1985) 7102.
36 C. M. Wernette and L. S. Kaguni, *J. Biol. Chem.*, 261 (1986) 14 764.
37 A. Spanos, R. Holliday and J. Gierman, *Hum. Genet.*, 73 (1986) 119.
38 E. Holler, H. Fischer, C. Weber, H. Stopper, H. Steger and H. Simek, *Eur. J. Biochem.*, 163 (1987) 297.
39 H. D. Simpson, T. Coolbear, M. Vermue and R. M. Daniel, *Biochem. Cell Biol.*, 68 (1990) 1292.
40 H. Lin, G. C. Cannon and S. Heinhorst, *Nucleic Acids Res.*, 18 (1990) 6659.
41 D. Filpula, P. A. Fisher and D. Korn, *J. Biol. Chem.*, 257 (1982) 2029.
42 J. A. Swack, E. Karawya, W. Albert, J. Fedorko, J. D. Minna and S. H. Wilson, *Anal. Biochem.*, 147 (1985) 10.
43 N. A. Timchenko, I. V. Shevelev, S. N. Drobchenko, O. P. Zhuchenko and V. M. Krutyakov, *Mol. Biol.*, 22 (1988) 780.
44 A. Brucker, L. A. Loeb and H. W. Thielmann, *Cancer Res.*, 50 (1990) 6894.
45 U. Bachrach and L. Abu-Elheiga, *Eur. J. Biochem.*, 191 (1990) 633.
46 M. Yamaguchi, T. Takahashi, K. Yasuda, Y. Shimura and A. Matsukage, *Eur. J. Biochem.*, 133 (1983) 277.
47 T. Ogura, N. Takenouchi, M. Yamaguchi, A. Matsukage, T. Sugimura and H. Esumi, *Biochem. Biophys. Res. Commun.*, 172 (1990) 377.
48 Z. T. Wang, J. Yang, Z. Q. Nie and M. Wu, *Biochemistry*, 30 (1991) 1127.
49 M. Y. W. T. Lee and N. L. Toomey, *Biochemistry*, 26 (1987) 1076.
50 M. J. Longley and D. W. Mosbaugh, *Methods Mol. Cell. Biol.*, 1 (1989) 79.
51 M. J. Longley, S. E. Bennett and D. W. Mosbaugh, *Nucleic Acids Res.*, 18 (1990) 7317.
52 M. J. Longley and D. W. Mosbaugh, *Biochemistry*, 30 (1991) 2655.
53 A. I. Scovassi, M. Stefanini and U. Bertazzoni, *J. Biol. Chem.*, 259 (1984) 10 973.
54 A. I. Scovassi, R. Izzo, E. Franchi and U. Bertazzoni, *Eur. J. Biochem.*, 159 (1986) 77.
55 C. F. Cesarone, A. I. Scovassi, L. Scarabelli, R. Izzo, M. Orunesu and U. Bertazzoni, *Cancer Res.*, 48 (1988) 3581.
56 M. Mezzina, A. Sarasin, N. Politi and U. Bertazzoni, *Nucleic Acids Res.*, 12 (1984) 5109.
57 M. Mezzina, E. Franchi, R. Izzo, U. Bertazzoni, J. M. Rossignol and A. Sarasin, *Biochem. Biophys. Res. Commun.*, 132 (1985) 857.
58 M. Mezzina, J. M. Rossignol, M. Philippe, R. Izzo, U. Bertazzoni and A. Sarasin, *Eur. J. Biochem.*, 162 (1987) 325.
59 M. Mezzina, J. Nardelli, S. Nocentini, G. Renault and A. Sarasin, *Nucleic Acids Res.*, 17 (1989) 3091.
60 U. Hübscher, G. Pedrali-Noy, B. Knust-Kron, W. Doerfler and S. Spadari, *Anal. Biochem.*, 150 (1985) 442.
61 K. Essani, R. Goorha and A. Granoff, *Gene*, 74 (1988) 71.
62 C. Bernelot-Moens and B. Demple, *Nucleic Acids Res.*, 17 (1989) 587.
63 D. Ramotar, S. C. Popoff and B. Demple, *Mol. Microbiol.*, 5 (1991) 149.
64 L. M. S. Chang, P. Plevani and F. J. Bollum, *J. Biol. Chem.*, 257 (1982) 5700.
65 P. S. Fitt, E. A. Fitt and H. Wille, *Biochem. J.*, 110 (1968) 475.
66 F. Hishinuma, K. Hirai and K. Sakaguchi, *Eur. J. Biochem.*, 77 (1977) 575.
67 M. F. Waters, A. G. McLennan and N. G. Carr, *J. Gen. Microbiol.*, 135 (1989) 2045.

68 Y. W. Rong and P. L. Carl, *Biochemistry*, 29 (1990) 383.
69 U. Bertazzoni, F. Lori, G. Achilli and E. Cattanco, *Clin. Chim. Acta*, 183 (1989) 101.
70 W. Luke, K. Hoefer, D. Moosmayer, P. Nickel, G. Hunsmann and K.-D. Jentsch, *Biochemistry*, 29 (1990) 1764.
71 G. Kraus, E. Behr, M. Baier, H. König and R. Kurth, *Eur. J. Biochem.*, 192 (1990) 207.
72 A. C. G. Porter, *Anal. Biochem.*, 117 (1981) 28.
73 H. S. Kim and T.-H. Liao, *Anal. Biochem.*, 119 (1982) 96.
74 E. Holler, H. Fischer and H. Simek, *Eur. J. Biochem.*, 151 (1985) 311.
75 E. Holler, H. Fischer, C. Weber, H. Stopper, H. Steger and H. Simek, *Eur. J. Biochem.*, 163 (1987) 397.
76 C. Weber, H. Fischer and E. Holler, *Eur. J. Biochem.*, 176 (1988) 199.
77 T. Yasuda, K. Mizuta, Y. Ikehara and K. Kishi, *Anal. Biochem.*, 183 (1989) 84.
78 K. O. Stratkvern and A. J. Raae, *Electrophoresis*, 11 (1990) 347.
79 F. Simonin, J.-P. Briand, S. Muller and G. de Murcia, *Anal. Biochem.*, 195 (1991) 226.
80 R. A. MacLaren, M. V. Witmer, E. Richardson and T. D. Stamoto, *Mutat. Res.*, 231 (1990) 265.
81 S. Seki, S. Ikeda, S. Watanabe, M. Matsushika, K. Tsutsui, K. Akiyama and B. Zhang, *Biochim. Biophys. Acta*, 1079 (1991) 57.
82 S. Seki, A. Takata, T. Nakamura, K. Akiyama and S. Watanabe, *Int. J. Biochem.*, 25 (1993) 53.
83 U. K. Laemmli, *Nature*, 227 (1970) 680.
84 S. A. Lacks and S. S. Springhorn, *J. Biol. Chem.*, 255 (1980) 7467.
85 S. A. Lacks, S. S. Springhorn and A. L. Rosenthal, *Anal. Biochem.*, 100 (1979) 357.
86 D. A. Hager and R. R. Burgess, *Anal. Biochem.*, 109 (1980) 76.
87 S. Seki and T. Oda, *Carcinogenesis*, 9 (1988) 2239.
88 S. Seki, S. Ikeda, K. Tsutsui and H. Teraoka, *Carcinogenesis*, 11 (1990) 1213.
89 E. M. Southern, *J. Mol. Biol.*, 98 (1975) 503.
90 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, 1989.
91 H. Towbin, T. Staehelin and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4350.
92 W. N. Burnette, *Anal. Biochem.*, 112 (1981) 195.
93 W. S. Dynan, J. J. Jendrisak, D. A. Hager and R. R. Burgess, *J. Biol. Chem.*, 256 (1981) 5860.
94 K. Ishii, T. Hasegawa, K. Fujisawa and T. Andoh, *J. Biol. Chem.*, 258 (1983) 12 728.
95 M. W. Hunkapiller, E. Lujan, F. Ostrander and L. E. Hood, *Methods Enzymol.*, 91 (1983) 227.
96 P. T. Matsudaira (Editor), *A Practical Guide to Protein and Peptide Purification for Microsequencing*, Academic Press, New York, 1989.