Enhancement of Immunocomplex Detection and Application to Assays for DNA Adduct of Benzo[a]pyrene

ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2003

Impact Factor: 5.64 · DOI: 10.1021/ac026204a · Source: PubMed

4 AUTHORS, INCLUDING:

Michael Weinfeld

University of Alberta

146 PUBLICATIONS **6,250** CITATIONS

SEE PROFILE

X. Chris Le

University of Alberta

248 PUBLICATIONS **7,753** CITATIONS

SEE PROFILE

Anal. Chem. **2003,** *75,* 247−254

Enhancement of Immunocomplex Detection and Application to Assays for DNA Adduct of Benzo[*a*]pyrene

Hailin Wang, Meiling Lu, Michael Weinfeld, and X. Chris Le*

Environmental Health Sciences Program, Department of Public Health Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2G3

The stability of antibody and formation of immunocomplexes are essential to high-sensitivity capillary electrophoresis immunoassays (CEIA). However, little attention has been paid to enhancing or maintaining immunocomplex formation and antibody stability to improve the performance of CEIA. We report here the use of nonspecific proteins, such as bovine serum albumin (BSA) and rabbit immunoglobulin (rIgG), to enhance immunocomplex formation and to stabilize antibodies and immunocomplexes for immunoassays. Complexes between DNA adducts of benzo[*a*]pyrenediol epoxide (BPDE) and their antibodies were examined using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). A tetramethylrhodamine (TMR)-labeled single-stranded oligonucleotide (16-mer) containing a single BPDE adduct was used as a fluorescent probe to study its immunocomplexes with a monoclonal antibody (8E11). To examine the formation of larger complexes, a TMR-labeled secondary antibody (anti-mouse), a primary antibody (mouse monoclonal antibody 5D11), and BPDE adducts in cellular DNA were used. We demonstrate that the use of nonspecific proteins stabilized the antibody and greatly enhanced the formation and stability of the immunocomplexes, resulting in substantial improvements in the detection limit (10-fold) and the reproducibility of the analysis. Another advantageous consequence of the stabilization was a 150-fold reduction of the concentration of the antibody needed for the immunoassay, resulting in reduced background and cost. We successfully applied this technique to the determination of DNA adducts of BPDE using a competitive immunoassay. The results from both small complexes (between a primary antibody and an oligonucleotide) and larger complexes (among a secondary antibody, a primary antibody, and cellular DNA) indicate that the technique can be extended to other immunoassays. We suggest that nonspecific proteins may assist the formation and stabilization of antibody−antigen complexes by maintaining the correct conformation of the antibody and antigen for optimum binding.

Immunoassays have been widely applied to clinical diagnosis, pharmaceutical developments, environmental chemistry, and the biological sciences. Capillary electrophoresis immunoassays (CEIA) offer additional advantages of efficient and rapid separation. Nielsen et al.[1] first used capillary zone electrophoresis (CZE) to separate human growth hormone (hGH), antibody against hGH, and the complex between these two proteins, demonstrating the feasibility of immunoassay using capillary electrophoresis (CE). Kennedy and co-workers developed the first competitive CEIA to detect insulin[2,3] and subsequently demonstrated continuous monitoring of insulin secretion in single Islets of Langerhans.[4,5] Shimura and Karger developed a noncompetitive immunoassay for three human growth hormone variants.[6]

With continued development over the past decade, capillary-electrophoresis-based immunoassays (CEIA) have been applied to the analysis of a variety of compounds, including drugs and metabolites,[7−21] hormones,[22,23] toxins,[24−26] peptides,[5,6] proteins,[27−33]

(1) Nielsen, R. G.; Richard, E. C.; Santa, P. F.; Sharknas, D. A.; Sittampalam, G. S. *J. Chromatogr.* **1991**, *539*, 177−185.
(2) Schultz, N. M.; Kennedy, R. T. *Anal. Chem.* **1993**, *65*, 3161−3165.
(3) Schultz, N. M.; Huang, L.; Kennedy, R. T. *Anal. Chem.* **1995**, *67*, 924−929.
(4) Tao, L.; Kennedy, R. T. *Anal. Chem.* **1996**, *68*, 3899−3906.
(5) Tao, L.; Aspinwall, C. A.; Kennedy, R. T. *Electrophoresis* **1998**, *19*, 403−408.
(6) Shimura, K.; Karger, B. L. *Anal. Chem.* **1994**, *66*, 9−15.
(7) Wan, Q. H.; Le, X. C. *Anal. Chem.* **2000**, *72*, 5583−5589.
(8) Ye, L. W.; Le, X. C.; Xing, J. Z.; Ma, M. S.; Yatscoff, R. *J. Chromatogr., B* **1998**, *714*, 59−67.
(9) Wan, Q. H.; Le, X. C. *J. Chromatogr., A* **1999**, *853*, 555−562.
(10) Wan, Q. H.; Le, X. C. *Anal. Chem.* **1999**, *71*, 4183−4189.
(11) Chen, F. T. A.; Evangelista, R. A. *Clin. Chem.* **1994**, *40*, 1819−1822.
(12) Liu, X.; Xu, Y.; Ip, M. P. C. *Anal. Chem.* **1995**, *67*, 3211−3218.
(13) Blais, B. W.; Cunningham, A.; Yamazaki, H. *Food Agric. Immunol.* **1994**, *6*, 409.
(14) Choi, J.; Kim, C.; Choi, M. J. *J. Chromatogr., B* **1998**, *705*, 277−282.
(15) Choi, J.; Kim, C.; Choi, M. J. *Electrophoresis* **1998**, *19*, 2950−2955.
(16) Thormann, W.; Lanz, M.; Caslavska, J.; Siegenthaler, P.; Portmann, R. *Electrophoresis* **1998**, *19*, 57−65.
(17) Ramseier, A.; Caslavska, J.; Thormann, W. *Electrophoresis* **1998**, *19*, 2956−2966.
(18) Caslavska, J.; Allemann, D.; Thormann, W. *J. Chromatogr., A* **1999**, *838*, 197−211.
(19) Zhang, X. X.; Li, J.; Gao, J.; Sun, L.; Chang, W. B. *Electrophoresis* **1999**, *20*, 1998−2002.
(20) Chiem, N. H.; Harrison, D. J. *Clin. Chem.* **1998**, *44*, 591−598.
(21) Wan, Q. H.; Le, X. C. *J. Chromatogr., B* **1999**, *734*, 31−38.
(22) Schmalzing, D.; Nashabeh, W.; Yao, X. W.; Mhatre, R.; Regnier, F. E.; Afeyan, N. B.; Fuchs, M. *Anal. Chem.* **1995**, *67*, 606−612.

* Corresponding author. Telephone: (780) 492-6416. Fax: (780) 492-7800. E-mail: xc.le@ualberta.ca.

10.1021/ac026204a CCC: $25.00
Published on Web 12/12/2002

and DNA lesions.[34−36] Despite the increasing applications of CEIA, there has been little attention paid to enhancing or maintaining immunocomplex formation to improve the performance of CEIA. Only a few studies applied fast separation or on-line reaction to prevent the dissociation of complexes during CE separation.[19,31] The formation and stability of immunocomplexes are key to the detection limits and reproducibility of immunoassays and are particularly crucial when dealing with trace levels of target analytes.

Three components are necessary for high sensitivity immunoassays using CE. They include the formation of immunocomplexes, efficient separation of immunocomplexes from antibody and antigen, and sensitive detection of the immunocomplexes and the unbound species. Several approaches have been developed for the separation of immunocomplexes. Free-zone CE,[1−5] capillary isoelectric focusing,[6] and nondenaturing sodium dodecyl sulfate (SDS) capillary gel electrophoresis[27,28] have been successful for a number of immunoassay applications. In addition, antibody and antigen have been modified with highly charged moieties (charge modulators) to further improve the separation of the antibody or antigen from the immunocomplexes.[37−39] For detection, laser-induced fluorescence and fluorescence polarization have shown excellent detection sensitivity. Although much work is focused on the separation and detection of immunocomplexes, there is no report on how to improve the formation and stability of trace levels of immunocomplexes.

The objective of this work was to enhance the formation of immunocomplexes and to stabilize antibodies and their complexes by using nonspecific proteins. We chose DNA adducts of benzo[*a*]pyrenediol epoxide (BPDE) as the target compounds because of their importance in carcinogenesis. The BPDE−DNA adduct is often used as a model for DNA damage in studies that investigate carcinogenesis, DNA repair, and genotoxicity. These require the determination of trace levels of BPDE−DNA adducts. We studied two binding systems to demonstrate both competitive and noncompetitive assay formats. The first involved binding of an antibody with a small oligonucleotide (16-mer) containing a single BPDE adduct. The other system involved the formation of larger complexes among a secondary antibody, a primary antibody, and BPDE−DNA adducts in cellular DNA. Studies of these binding systems using different antibodies demonstrated that nonspecific proteins, such as bovine serum albumin (BSA) and immunoglobulin (IgG), were able to markedly improve the immunoassays.

EXPERIMENTAL SECTION

Reagents. Antibodies 5D11 and 8E11 were purchased from BD Pharmingen (Mississauga, ON). Polyclonal rabbit IgG antibody (rIgG) and goat anti-mouse IgG (heavy- and light-chain TMR conjugated) were purchased from Calbiochem (La Jolla, CA). Bovine serum albumin (BSA) and single-stranded DNA binding protein were obtained from Sigma (St. Louis, MO). Solvents and other biochemicals were supplied by Sigma (Oakville, ON, Canada) and Fisher Scientific (Nepean, ON, Canada).

Instrumentation. A laboratory-built capillary electrophoresis laser-induced fluorescence (CE-LIF) system was used as previously described.[40] Briefly, electrophoresis was driven by a high-voltage power supply (CZE 1000R, Spellman High Voltage Electronics, Plainview, NY). Separation conditions, including sample injection time and voltage, separation voltage and run time, were controlled by a LabView (National Instruments, Austin, TX) program run on a Macintosh computer. Capillaries used for these experiments were uncoated fused silica, with a 20-μm i.d., 150-μm o.d., and 30-cm length (Polymicro Technologies, Phoenix, AZ). The injection end of the capillary was placed in sample solution or running buffer, along with an electrode connected to the high voltage power supply. The other end of the capillary was inserted to a 2-cm sheath-flow cuvette (2-mm square quartz, 0.9-mm wall thickness, and 200 μm × 200 μm square inner bore) (NSG Precision Cells, NY). A 5-mW green helium−neon laser (Melles Griot, Irvine, CA) with an excitation wavelength of 543.5 nm was focused by a 10× (N.A. 0.25) microscope objective (Melles Griot) onto the sheath flow cuvette just below the capillary end. Fluorescence was collected at 90° to the direction of excitation by a 60× (N.A. 0.7) microscope objective (LWD-M Plain, Universe Kogaku, Japan). The transmitted light was spectrally filtered with a 580DF40 band-pass filter and detected by an R1477 photomultiplier tube (PMT) (Hamamatsu Photonics, Japan). The PMT signal was transferred to the interface box and digitized by the input/output board. Data were collected at a sampling rate of 10 Hz.

Procedures. TMR-labeled 16-mer oligonuleotide with the sequence 5′-CCCATTATGCATAACC-3′ was synthesized by University Core DNA Services, University of Calgary (Alberta, Canada). (±)-anti-*r*-7,*t*-8-Dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(±)-anti-BPDE] was supplied by the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). The (±)-anti-BPDE and the TMR-16-mer were used to synthesize the BPDE-N^2 deoxyguanosine (dG) adduct.[41,42] Detailed protocols for the preparation and purification of this TMR−BPDE−16-mer have

(23) Schmalzing, D.; Nashabeh, W.; Fuchs, M. *Clin. Chem.* **1995**, *41*, 1403−1406.
(24) Driedger, D. R.; LeBlanc, R. J.; LeBlanc, E. L.; Sporns, P. *J. Agric. Food Chem.* **2000**, *48*, 1135−1139.
(25) Lam, M. T.; Wan, Q. H.; Boulet, C. A.; Le, X. C. *J. Chromatogr., A* **1999**, *853*, 545−553.
(26) Lam, M. T.; Boulet, C. A.; Le, X. C. *Anal. Chim. Acta* **2002**, *457*, 21−28.
(27) Qu, J. P.; Wang, Q. G.; Cheung, T. M.; Chan, S. T. H.; Yeung, W. S. B. *J. Chromatogr., B* **1999**, *727*, 63−71.
(28) Qu, J. P.; Chan, S. T. H.; Yeung, W. S. B. *J. Chromatogr., B* **1999**, *731*, 389−394.
(29) Riechel, P.; Weiss, T.; Weiss, M.; Ulber, R.; Buchholz, H.; Scheper, T. *J. Chromatogr., A* **1998**, *817*, 187−193.
(30) Phillips, T. M.; Dickens, B. F. *Electrophoresis* **1998**, *19*, 2991−2996.
(31) German, I.; Buchanan, D. D.; Kennedy, R. T. *Anal. Chem.* **1998**, *70*, 4540−4545.
(32) Pavski, V.; Le, X. C. *Anal. Chem.* **2001**, *73*, 6070−6076.
(33) Schmerr, M. J.; Jenny, A. *Electrophoresis* **1998**, *19*, 409−414.
(34) Le, X. C.; Xing, J. Z.; Lee, J.; Leadon, S. A.; Weinfeld, M. *Science* **1998**, *280*, 1066−1069.
(35) Carnelley, T. J.; Barker, S.; Wang, H.; Tan, W.; Weinfeld, M.; Le, X. C. *Chem. Res. Toxicol.* **2001**, *14*, 1513−1522.
(36) Tan, W.; Carnelley, T. J.; Murphy, P.; Wang, H.; Lee, J.; Barker, S.; Weinfeld, M.; Le, X. C. *J. Chromatogr., A* **2001**, *924*, 377−386.
(37) Evangelista, R. A.; Chen, F. T. A. *J. Chromatogr., A* **1994**, *680*, 587−591.
(38) Chen, F. T. A. *J. Chromatogr., A* **1994**, *680*, 419−423.
(39) Fuchs, M.; Nashabeh, W.; Schmalzing, D. Composition, method and apparatus for ultrafast electroseparation analysis; U.S. Patent 5,630,924, 1997.
(40) Le, X.; Scaman, C.; Zhang, Y.; Zhang, J.; Dovichi, N. J.; Hindsgaul, O.; Palcic, M. M. *J. Chromatogr., A* **1995**, *716*, 215−220.
(41) Margulis, L. A.; Ibanez, V.; Geacintov, N. E. *Chem. Res. Toxicol.* **1993**, *6*, 59−63.
(42) Funk, M.; Seidel, A.; Jernstrom, B. *Bioconjugate Chem.* **1997**, *8*, 310−317.

been described previously.[35] The TMR-labeled BPDE−16-mer oligonucleotide was used as a fluorescent probe for studies of its binding with antibodies and for competitive immunoassays of BPDE−DNA adducts.

Cellular DNA containing BPDE−DNA adducts was obtained from A549 human lung carcinoma cells as described previously.[35,36] The cells were maintained in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum. The cells were seeded at 1×10^5 cells/plate and maintained at 95% humidity and 5% CO_2 for 20 h prior to the addition of BPDE. After replacing with the media containing BPDE, the cells were further incubated for 2 h. The cells were then washed with phosphate buffered saline (PBS) prior to the addition of DNAzol lysis reagent (Gibco BRL) to facilitate cell lysis and DNA extraction. Subsequent steps involved an ice-cold 99.9% ethanol precipitation and a cold 70% ethanol wash to purify the genomic DNA. The final DNA pellet was dissolved in distilled deionized water (ddH_2O), and the DNA concentration was measured at OD_{260} using ddH_2O as a blank. An aliquot of the DNA sample was heat-denatured at 95 °C followed by cooling on ice. An aliquot of the denatured DNA was used for the analysis of BPDE−DNA adducts.

Samples were electrokinetically injected into the capillary using an injection voltage of 15 kV for 5 s. The separation was carried out at room temperature with a separation voltage of 15 kV. The running buffer was 1× Tris−glycine (25 mM Tris, 192 mM glycine, pH 8.3). The capillary was regularly washed after three sample injections with 0.02 M NaOH electrophoretically (15 kV for 7 min), followed by electrophoresis using water and 1× Tris−glycine buffer (pH 8.3) for 7 min. All capillary electrophoresis data were analyzed using IgorPro software (version 3.1, WaveMetrics, Lake Oswego, OR).

Enhancement of Antibody−Substrate Complex Formation by Nonspecific Proteins. Stock solutions (2 mg/mL) of BSA and rIgG were dissolved in CE running buffer (25 mM Tris and 192 mM glycine, pH 8.3) and were kept at 4 °C. To perform enhancement experiments, BSA (100 μg/mL) or rIgG (100 μg/mL) was first mixed with 24 nM TMR−BPDE−16-mer in the Tris−glycine buffer and then with the antibody 8E11 (13−67 nM). The sample mixture was analyzed by CE-LIF after incubation for 5 min at room temperature (21 °C). Controls without addition of BSA or rIgG were prepared similarly. Unless stated otherwise, the antibody was added last to the sample solutions. This was to prevent possible loss of antibody activity before immunocomplex formation. All samples were prepared in polymerase chain reaction (PCR) tubes (Rose Scientific, Edmonton, Alberta). These tubes were made of ultrapure polypropylene and did not readily adsorb proteins.

To examine whether the change of sample matrix (the addition of nonspecific proteins) could be responsible for the enhancement of the complex signal in the presence of nonspecific proteins, the following experiments were performed: rIgG (0.7 μM) was added to three identical mixtures containing antibody 8E11 (13 nM) and TMR−BPDE−16-mer (12 nM) immediately or 15 and 50 min after the mixing of the antibody with the TMR−BPDE−16-mer. These samples were incubated for 5 min after addition of rIgG, then analyzed by CE-LIF.

Stability of Mouse Monoclonal Antibody 8E11. Three stock solutions of antibody 8E11 (~70 nM) were prepared in Tris−glycine buffer only or supplemented with 0.14 μM or 0.7 μM rIgG. These solutions were incubated at room temperature (21 °C). Stability of the antibody 8E11 in these solutions (with or without rIgG) was monitored over time for its binding activity with TMR−BPDE−16-mer. After various periods of incubation, 1.0 μL of each antibody 8E11 solution was mixed with 19 μL TMR−BPDE−16-mer solution. The samples were diluted in the Tris−glycine buffer (pH 8.3) containing 0.7 μM rIgG and were analyzed by CE-LIF after incubation for 5 min at room temperature. The concentrations of the TMR−BPDE−16-mer and antibody 8E11 in the final mixtures were 50 and 3.5 nM, respectively. Although the antibody can form 1:1 and 1:2 complexes with the TMR−BPDE−16-mer, the complex of one antibody with two TMR−BPDE−16-mer molecules (1:2 complex) is favored in the presence of a limiting amount of the antibody.[43] The fluorescence intensity of the 1:2 complex was used to determine the activity of the antibody 8E11.

Stability of the Complexes. Three samples containing 24 nM TMR−BPDE−16-mer and 13 nM 8E11 were prepared in the Tris−glycine buffer only or in the Tris−glycine buffer supplemented with 1.5 μM rIgG or 1.5 μM BSA. The samples were analyzed by CE-LIF after incubation for 5, 30, 60, 90, and 120 min at room temperature (21 °C). The peak areas of the two complexes (1:1 and 1:2 stoichiometry) were measured, and the sum was used to evaluate the complex stability over time.

Effect of Nonspecific Protein Concentration. While TMR−BPDE−16-mer and the antibody 8E11 were kept constant at 24 nM and 13 nM, respectively, the concentration of rIgG was varied from 0.07 μM (10 μg/mL) to 1.4 μM (200 μg/mL). The samples were analyzed by CE-LIF after incubation for 10 min at room temperature. For BSA, its concentration was varied from 25 μg/mL (0.4 μM) to 1200 μg/mL (18 μM), while TMR−BPDE−16-mer and the antibody 8E11 were kept at 24 nM and 13 nM, respectively. The samples were analyzed after incubation for 1 h at room temperature. The peak areas of the two complexes (1:1 and 1:2 stoichiometry) were measured, and the sum was used to evaluate the effect of the nonspecific protein on the complex formation.

Competitive Immunoassays for BPDE−DNA Adducts. Competitive immunoassays were carried out in a manner similar to that as previously described.[36] The TMR-labeled BPDE−16-mer oligonucleotide was used as the probe (competitor). For the competitive assay in the presence of rIgG, the mixtures containing 60 nM the oligonucleotide probe, 0.7 μM rIgG, 2.7 nM mouse monoclonal antibody 8E11, and 80 μg/mL of the DNA from A549 cells were incubated in 20 μL of Tris−glycine buffer (pH 8.3) at room temperature for 30 min. Samples were subjected to CE-LIF analysis to detect both free and antibody-bound fluorescent probes.

RESULTS

Stabilization of Complexes between Antibody and BPDE Oligonucleotide. The mouse anti-BPDE monoclonal antibody, MAb 8E11, specifically recognizes BPDE−DNA adducts and has very low cross-reaction with undamaged DNA.[35] We chose the binding between this antibody and BPDE−DNA adducts as a

(43) Wang, H.; Xing, J.; Tan, W.; Lam, M.; Carnelley, T.; Weinfeld, M.; Le, X. C. *Anal. Chem.* **2002**, *74*, 3714−3719.

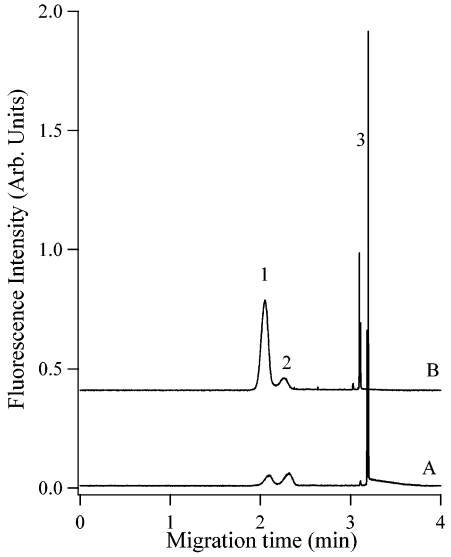

Figure 1. CE-LIF analysis of immunocomplexes between antibody 8E11 (67 nM) and TMR-labled BPDE−16-mer oligonucleotide (24 nM) in the absence (A) and presence (B) of bovine serum albumin (100 μg/mL, 1.5 μM). Peak 1 corresponds to the complex of one antibody binding with one BPDE−16-mer (1:1 stoichiometry). Peak 2 corresponds to the complex of one antibody binding with two BPDE−16-mer molecules (1:2 stoichiometry). Peak 3 represents the unbound oligonucleotide. A Tris−glycine (25 mM Tris and 192 mM glycine) buffer at pH 8.3 was used for sample preparation and CE separation. A fused-silica capillary (20-μm i.d. × 150-μm o.d., 30-cm long) was used for separation at an applied voltage of 15 kV.

model system of assays for DNA damage to demonstrate the improvement of the detection limits and the enhancement of the formation and stability of immunocomplexes using nonspecific proteins. Figure 1 compares immunocomplexes formed in the presence and absence of a nonspecific binding protein, bovine serum albumin (BSA). The 16-mer oligonucleotide contained a single BPDE adduct and was labeled at the 5′ end with a tetramethylrhodamine (TMR). The mouse monoclonal antibody (8E11) was able to form 1:1 and 1:2 complexes with the oligonucleotide because the antibody was bidentate.[43] The two complexes were well-resolved from each other as peak 1 (1:1 complex) and peak 2 (1:2 complex) and were separated from the unbound oligonucleotide (peak 3). The complex of one antibody binding with one oligonucleotide was dominant (peak 1) because the antibody concentration was three times that of the oligonucelotide. In the absence of BSA (bottom trace in Figure 1), only small amounts of the complexes (peaks 1 and 2) were formed between the antibody 8E11 and the 16-mer, leaving most of the 16-mer oligonucleotide unbound (peak 3). In the presence of 100 μg/mL (~1.5 μM) BSA, the level of the 1:1 complex (peak 1) was increased about 5-fold, with a corresponding decrease of the unbound 16-mer (top trace in Figure 1). Control experiments showed that there was no complex between BSA and the 16-mer.

We also used TMR-labeled deoxyuraciltriphosphate (dUTP) as an internal standard to confirm that the enhancement is due to the enhanced complex formation rather than an enhancement of TMR fluorescence per se. The dUTP was labeled with TMR at the 5′ end and it did not bind with the anti-BPDE antibody. TMR-dUTP migrated between the complexes and the unbound TMR−BPDE−16-mer. There was no change of the fluorescence intensity of the internal standard in the presence of BSA, but the complex signal was increased 5-fold (data not shown). These results demonstrate that BSA can enhance the formation of complexes between the antibody and the BPDE adducts in the oligonucleotide.

Similar improvements were also observed with rabbit immunoglobulin G (rIgG). In the presence of 100 μg/mL (~0.7 μM) rIgG in the mixture of 8E11 antibody (13 nM) and BPDE−16-mer (12 nM), the complex signals increased about 5-fold, with the corresponding decrease of the unbound 16-mer oligonucleotide. In addition, a number of other nonspecific proteins, such as human IgG, human serum albumin, human γ homoglobulin, and gelatin of type A and B, could also enhance the immunocomplex peaks. However, adsorption of gelatin on the surface of the capillary inner wall was severe, resulting in observable changes in separation efficiency. Therefore, no further study was carried out using gelatin. We chose to use rIgG and BSA for the remainder of the study.

Several possible explanations may be considered for the observed enhancement of immunocomplex detection. These include the stabilization of the antibody, the enhancement of complex formation, the stabilization of the complexes, the reduction of protein adsorption on the sample vial, and alterations to CE separation and LIF detection. Sample vials were those used for polymerase chain reaction (PCR). They had a smooth and inert surface and were not known to adsorb proteins. Furthermore, we measured the concentration of 10 μg/mL mouse IgG stored in the PCR sample vials. We did not observe a decrease of the protein concentration over a 2-h incubation period. This confirms that the adsorption of proteins on these sample vials was negligible during the sample incubation period.

To examine whether alterations (interference) to CE-LIF due to the change of sample matrix (the addition of nonspecific proteins) could be responsible for the observed increase in complex signal intensity, we carried out the following experiments: To three identical mixtures of antibody 8E11 and BPDE−16-mer, the same amount of rIgG was added either immediately or 15 and 50 min after the mixing of the antibody with the BPDE−16-mer. To avoid the activity loss in dilute solution before the antibody reacts with the BPDE−16-mer, the antibody was added to the solutions after the addition of the BPDE−16-mer. Figure 2 shows electropherograms from the CE-LIF analysis of these incubation mixtures. Only small complex concentrations were detected in the absence of rIgG (Figure 2A). A 5-fold increase of the complexes was observed when 0.7 μM rIgG was incubated with the antibody 8E11 and BPDE−16-mer (Figure 2B), consistent with the results from the use of BSA for enhancement of complexes. In two other parallel experiments, the rIgG was added to the mixture 15 and 50 min after the antibody 8E11 and BPDE−16-mer were incubated. Although the same amount of rIgG (0.7 μM) was present, the addition of rIgG 50 min after the mixing of antibody 8E11 with BPDE−16-mer did not substantially enhance the complex formation (Figure 2D). These results suggest that the observed increases of complex peaks are not due to the changes of sample matrix by the addition of rIgG. It is evident that the enhancement depends on what order (sequence) the nonspecific protein is mixed with the antibody and the BPDE−16-mer. The highest enhancement was achieved when the

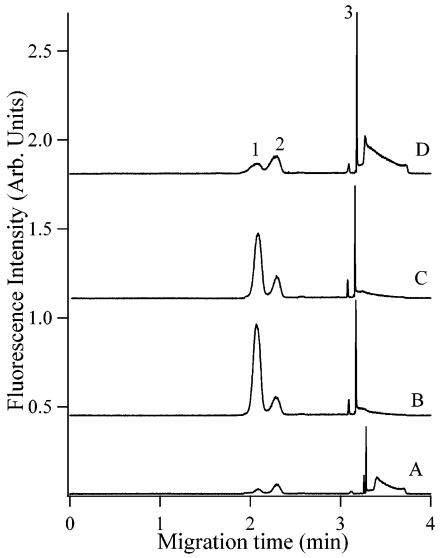

Figure 2. Electropherograms from the analysis of the immunocomplexes between antibody 8E11 (13 nM) and TMR-labeled BPDE−16-mer oligonucleotide (12 nM) in the absence (A) and presence (B−D) of rabbit IgG (100 μg/mL, 0.7 μM). In (B), rabbit IgG was added together with the antibody 8E11 and the BPDE−16-mer. In (C) and (D), antibody 8E11 and the BPDE−16-mer were incubated for 15 (C) and 50 min (D) before the addition of the rabbit IgG. The CE-LIF conditions were the same as in Figure 1.

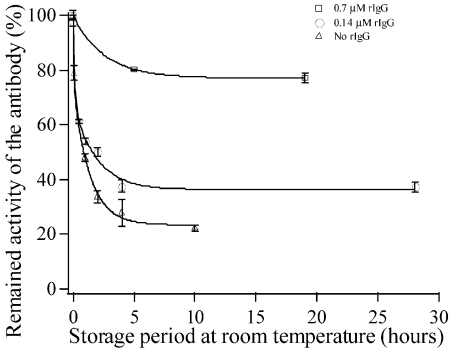

Figure 3. Activity of mouse monoclonal antibody (MAb) 8E11 in the absence or presence of nonspecific protein rIgG. The antibody 8E11 (70 nM) was kept at room temperature either in 25 mM Tris−192 mM glycine (pH 8.3) without nonspecific protein (△) or in the Tris−glycine buffer supplemented with 0.14 μM (○) or 0.7 μM (□) of rIgG. After a determined storage period, 1 μL of the antibody solution was mixed with 19 μL of the BPDE−16-mer solution to make a final solution containing 3.5 nM antibody 8E11 and 50 nM BPDE−16-mer. The complex of one antibody with two TMR−BPDE−16-mer molecules (1:2 complex) was favored because of the presence of the excess TMR−BPDE−16-mer and a limiting amount of the antibody. The fluorescence intensity of the 1:2 complex was used to determine the activity of the antibody 8E11. Results were from analyses of triplicate sample preparations. CE-LIF conditions were the same as in Figure 1.

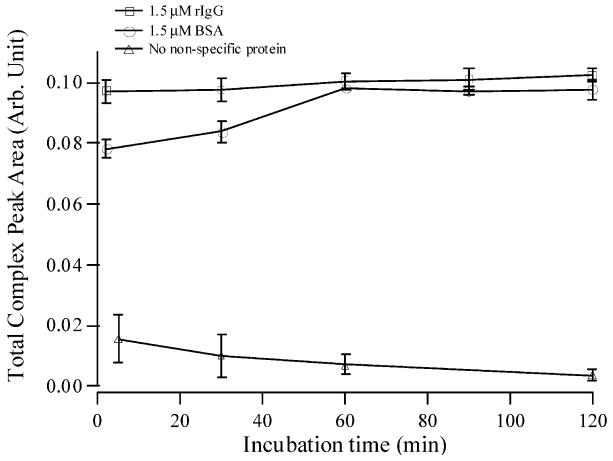

Figure 4. Stability of the immunocomplexes between antibody 8E11 (13 nM) and TMR-labeled BPDE−16-mer oligonucleotide (24 nM) in the absence or presence of nonspecific proteins. Three samples containing 24 nM TMR−BPDE−16-mer and 13 nM 8E11 were prepared either in 25 mM Tris−192 mM glycine (pH 8.3) without nonspecific protein (△) or in the Tris−glycine buffer supplemented with 1.5 μM BSA (○) or 1.5 μM rIgG (□). Peak areas of both complex peaks (1:1 and 1:2 stoichiometry) were measured and were combined. Results were from analyses of triplicate sample preparations. CE-LIF conditions were the same as in Figure 1.

nonspecific protein is added to the BPDE−16-mer solution before the addition of the antibody. The enhancement of the complexes detected is probably due to a combination of the stabilization of the antibody, the enhancement of complex formation, and the stabilization of the complexes. These possibilities were further explored as follows.

Stabilization of antibodies by BSA and rIgG was confirmed by examining immunocomplexes with trace amounts of antibody 8E11. Figure 3 shows the activity of dilute antibody over a storage period of 30 h in the absence and presence of rIgG. This was determined indirectly by monitoring the intensity of the 1:2 complex between the antibody and the BPDE−16-mer. In this experiment, the oligonucleotide concentration (50 nM) was in large excess, and the antibody concentration (3.5 nM) was limited, a condition favoring the formation of the 1:2 complex, provided that active antibody was present. Therefore, the amounts of the 1:2 complex formed represent the amounts of active antibody. In the presence of 0.7 μM rIgG, trace amounts of antibody 8E11 were found to be relatively stable for 20 h, maintaining at least 80% of their activity. In the absence of the stabilizing protein, antibody 8E11 in Tris−glycine buffer gradually lost its activity, with only 25% remaining after 4 h.

Enhancement of the complex signals by the use of BSA and rIgG was observed for a range of antibody and BPDE−16-mer concentrations. For example, using different concentrations of antibody (17, 33, and 67 nM) and a constant concentration of BPDE−16-mer (24 nM), complex signals were consistently enhanced by 4−5-fold in the presence of BSA (data not shown).

In addition to enhancement of complex signals, the use of nonspecific proteins was found to stabilize the complexes for more than 2 h (Figure 4). Evidence was obtained by monitoring the stability of the complexes over time. In the absence of nonspecific protein, the complexes gradually dissociated, especially at lower concentrations of the antibody. For example, with 17 nM of antibody 8E11 and 24 nM BPDE−16-mer, >75% of the complexes dissociated after 2 h (Figure 4). As a consequence of the gradual dissociation of the complexes, measurements of complex peak areas gave a relative standard deviation (RSD) of 28% (n = 10). In the presence of BSA, the RSD was reduced to 3.7% (n = 10). The stabilization of the complexes resulted in improved reproducibility.

The effect of protein concentration on the detection of immunocomplexes is shown in Figure 5. The total peak area of the

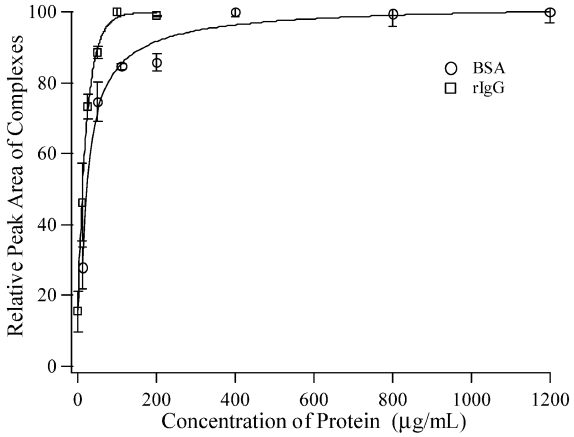

Figure 5. Effect of the concentration of nonspecific proteins on the intensity of complexes between antibody 8E11 and TMR-labeled BPDE−16-mer. Various concentrations of BSA (25−1200 μg/mL or 0.4−18 μM) and rabbit IgG (10−200 μg/mL or 0.07−1.4 μM) were incubated with antibody 8E11 (13 nM) and BPDE−16-mer (24 nM) for 10 min before the mixtures were analyzed by CE-LIF. The same CE-LIF conditions as shown in Figure 1 were used. Peak areas of both complex peaks (1:1 and 1:2 stoichiometry) were measured and were combined (n = 3).

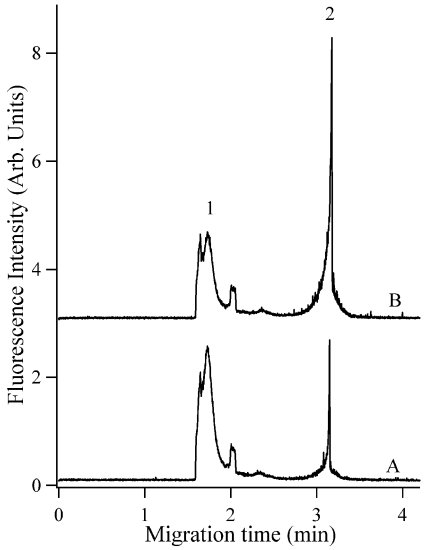

Figure 6. CE-LIF analysis of immunocomplexes between a fluorescent secondary antibody (anti-mouse IgG), a primary mouse monoclonal antibody (5D11), and BPDE−DNA adducts from A549 cellular DNA. The primary mouse monoclonal antibody 5D11 (27 nM) was incubated in Tris−glycine buffer for 60 min either without (A) or with (B) the presence of rabbit IgG (100 μg/mL, 0.7 μM). These were subsequently incubated with the secondary antibody (goat anti-mouse) and the DNA for 2 h prior to CE-LIF analysis. Peak 2 represents the tertiary complex between the secondary antibody, primary antibody, and BPDE−DNA adducts. Peak 1 is due to the excess of secondary antibody and the complex between the secondary and primary antibodies. CE-LIF conditions were the same as in Figure 1.

complexes formed between antibody 8E11 (13 nM) and BPDE−16-mer (24 nM) was measured. Various concentrations of BSA (25−1200 μg/mL or 0.4−18 μM) and rIgG (10−200 μg/mL or 0.07−1.4 μM) were added to the reaction mixture of the antibody 8E11 and BPDE−16-mer. The intensity of immunocomplexes increased with increasing amounts of BSA up to 400 μg/mL (6 μM) or rIgG up to 100 μg/mL (0.7 μM), at which point the intensity of the complex peaks represented an increase of 5-fold compared with the absence of BSA or rIgG.

Interestingly, the maximum detection of the complexes was achieved at a lower concentration of rIgG (~10-fold lower in molar concentration) than BSA. Furthermore, with the addition of rIgG, the reaction between antibody 8E11 and BPDE−16-mer could reach equilibrium within minutes (see Figure 4). Thus, in the presence of rIgG, only a 5-min incubation of the antibody with the target BPDE−16-mer was sufficient prior to CE-LIF analysis.

Larger Complexes between Secondary Antibody, Primary Antibody and DNA Adducts in Cellular DNA. We extended the strategy of enhancing the complex detection to immunoassays of DNA adducts in genomic DNA. Determination of DNA adducts in cellular DNA is achieved by using a primary antibody (mouse monoclonal) that recognizes the damage and a fluorescently labeled secondary antibody (anti-mouse IgG) that serves as a detection probe. Figure 6 shows typical electropherograms from the CE-LIF analyses of cellular DNA samples containing BPDE−DNA adducts. The DNA adducts and the mouse monoclonal antibody (5D11) against the DNA adducts were not fluorescent. The secondary antibody (goat anti-mouse IgG) was labeled with tetramethylrhodamine (TMR) and served as an affinity probe. The DNA adducts were bound by the primary antibody, which in turn complexed with the fluorescent secondary antibody. Figure 6 shows that the tertiary complex (peak 2) was well-resolved from the excess amounts of the secondary antibody and the complex between the secondary and primary antibodies (peak 1). It is clear that the addition of rIgG (0.7 μM) markedly enhanced the complex (peak 2 in Figure 6B), while there was no change to the background noise.

Application to Immunoassays. To demonstrate an application of the immunocomplex enhancement effect, we have compared competitive immunoassay of BPDE−DNA adducts in the presence and absence of nonspecific proteins. With the competitive immunoassay approach, BPDE−DNA adducts in cell samples compete with the TMR-labeled BPDE−16-mer fluorescent probe for the limiting amount of antibody. The binding of the BPDE−DNA adducts with the antibody results in increases in the free TMR−BPDE−16-mer probe and decreases in the antibody-bound probe. The intensity of the complexes between the antibody and BPDE−DNA adducts can be evaluated from the ratio of the free to bound fluorescent probe (F/B). Figure 7 shows dose−response curves from the analysis of BPDE−DNA adducts in A549 cells. The analyses were carried out with or without the use of rIgG for immunocomplex enhancement. The sensitivity can be estimated from the slope of the dose−response curve at low concentration.[44] The slopes of the two dose−response curves indicate an improvement in sensitivity of 10-fold.

In the absence of nonspecific protein, a relatively high concentration of antibody 8E11 (60 μg/mL or 400 nM) was needed to perform the assay. The corresponding assay was able to detect the BPDE−DNA adducts in A549 cells that were treated with 18.8 μM of BPDE.[36] In the presence of human IgG, the concentration of antibody 8E11 needed was 150-fold lower (0.4 μg/mL or 2.7

(44) *Immunoassays*. Gosling, J. P., Ed; Oxford University Press: Oxford, U.K., 2000.

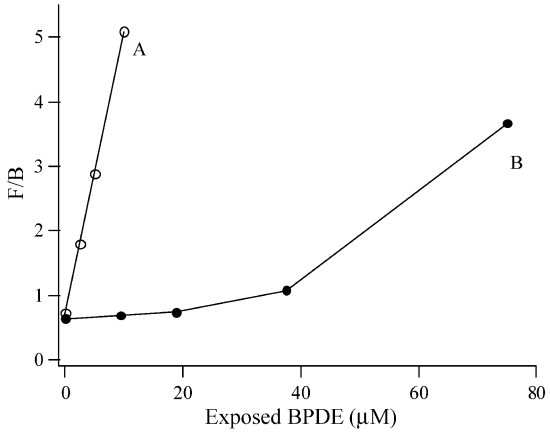

Figure 7. Dose−response curves of BPDE−DNA adducts from the CE-LIF competitive immunoassay of BPDE−DNA adducts in A549 cells that were incubated with various concentrations of BPDE. (A) rIgG was used in the immunoassay. (B) No rIgG was used. BPDE−DNA adducts in the samples compete with the TMR−BPDE−16-mer fluorescent probe for the limiting amount of antibody, resulting in increases in the free TMR−BPDE−16-mer probe and decreases in the antibody-bound probe. F/B represents the ratio of peak area of the free to bound TMR−BPDE−16-mer probe.

nM). The dramatic reduction of antibody reduced not only the cost but also the background, thereby improving detection limit. The competitive immunoassay was able to detect trace amount of BPDE−DNA adducts from A549 cells that was treated with BPDE at a concentration as low as 2 μM.

DISCUSSION

The above results clearly demonstrate that nonspecific proteins (e.g., BSA and rIgG) can improve capillary electrophoresis immunoassays (CEIA). To understand the reasons for this improvement, we have considered several possibilities. First, the use of nonspecific proteins may prevent adsorption of analytes on the sample vials and the capillary, resulting in an enhancement of analyte signals. Second, nonspecific proteins may stabilize antibodies through weak interactions. Third, nonspecific proteins may assist the formation and stabilization of antibody−antigen complexes by maintaining the correct conformation of the antibody and antigen for optimum binding. Our results support the latter two hypotheses, as discussed below.

The nonspecific proteins BSA and nonfat dried milk have been widely used as blockers in enzyme-linked immunosorbent assay (ELISA) and western blotting to eliminate the nonspecific binding sites on solid phases, such as microtiter plates, membranes, microspheres, and tubes.[44,45] We considered the question of whether the nonspecific proteins BSA and rIgG in the CE immunoassays also serve as blocking agents to eliminate the adsorption of the analytes onto the uncoated fused-silica capillary surface. In ELISA and western blotting, a special solid material with strong nonspecific binding, such as nitrocellulose, is typically used to immobilize target antibody or antigen. After immobilization of antibody or antigen, the excessive binding sites on the solid phase must be blocked. Otherwise, the detection probes would be adsorbed onto the solid phase, resulting in background levels too high to detect trace levels of target antibody/antigen. However, the CEIA approach reported here is very different from ELISA and western blotting. In CEIA, the fused-silica capillary is used for electrophoretic separation, and it usually has weak nonspecific binding. To estimate the extent of adsorption, we monitored the fluorescent compounds eluted with 0.1 M NaOH after a typical CE analysis. The adsorbed compounds from the preceding CE analysis were eluted with NaOH and detected with laser-induced fluorescence. We observed that the fluorescent signal corresponding to the adsorption was <5% of the total fluorescence for the preceding CE analysis. When the nonspecific proteins were added to the samples, there was no pronounced decrease in adsorption of the fluorescent compounds. In addition, we did not observe marked adsorption of proteins on the sample vials.

Furthermore, the improvement by use of nonspecific proteins depended upon when the nonspecific proteins were added to the antibody and antigen solutions (Figure 2). In the presence of the same amounts of the nonspecific proteins in the sample mixtures for CE analysis, the desired enhancement of complexes was achieved only when the nonspecific proteins were mixed immediately with the antibody and the BPDE−16-mer. These results suggest that blocking adsorption by nonspecific proteins is unlikely a main reason accounting for the observed improvement of CEIA results.

We suggest that the main reason for the improvement of CEIA by nonspecific proteins is due to the stabilization of the antibody and antibody−antigen complexes. This may come from weak nonspecific interactions between nonspecific proteins and antibodies and between nonspecific proteins and DNA (oligonucleotides). It is possible that the interaction of nonspecific proteins with the antibody stabilizes the antibody and maintains the correct antibody conformation for binding with the BPDE−16-mer. To provide stabilization of a protein molecule, the ratio of hydrophobic and hydrophilic regions on its surface is important.[46] Changes in this ratio can cause a rearrangement of hydrogen bonds that results in conformation changes of the whole molecule.[46] When the antibody is substantially diluted in aqueous buffer solutions, the ratio of hydrophobic and hydrophilic regions may change, leading to possible denaturation of the antibody and reduction in binding activity. In the presence of nonspecific proteins in the dilute solutions, the natural conformation of the antibody may be maintained. The interaction of nonspecific proteins with the antibody is probably the reason for the stabilization of the antibody.

Maintaining the conformation of the antigen (and epitope) for optimum binding with the antibody is another possible effect of the nonspecific protein. Presumably the BPDE moiety in the DNA oligonucleotide needs to be exposed (not hidden in the DNA structure) in order for the anti-BPDE antibody to bind with it. Thus, we compared the effects of nonspecific proteins and a specific single-stranded DNA binding (SSB) protein on the formation of an antibody complex with the BPDE-oligonucelotide. These proteins have various affinities with the oligonucleotide (binding constants range from 10^4 M^{-1} for the nonspecific proteins to 10^7 M^{-1} for the SSB protein).

(45) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Lab Press: Plainview, NY, 1989.

(46) Melnikova, Ya. I.; Odintsov, S. G.; Kravchuk, Z. I.; Martsev, S. P. *Biochemistry (Moscow)*, **2000**, *65* (11), 1256.

There have been reports on the weak interactions between serum albumin, immunoglobulin and DNA/(oligonucleotide).[47] These weak, reversible interactions between the nonspecific proteins and the DNA may help to stabilize the conformation of the DNA. It is possible that these interactions keep the epitope BPDE exposed for antibody binding. It has been shown that nonspecific IgG has a stronger affinity for oligonucleotides (binding constant $\sim 1.6 \times 10^5\ M^{-1}$) than serum albumin (binding constant $< 5 \times 10^4\ M^{-1}$)[47] and has a stronger ability to induce conformation change or maintain the conformation of DNA. This is consistent with our observations that rIgG works better than BSA in enhancing complex formation between anti-BPDE antibody and BPDE-oligonucleotide. A smaller amount of rIgG than BSA was needed to enhance the complex formation, and a faster complex formation was achieved with rIgG (Figure 5).

Interestingly, further increase of the binding affinity between the nonspecific protein and the oligonucleotide did not enhance the formation of the specific complexes between the anti-BPDE antibody and the BPDE−16-mer. This was observed by using a stronger DNA binding protein, single-stranded DNA binding (SSB) protein, which has binding constants of $5-23 \times 10^6\ M^{-1}$ for single-stranded oligonucleotides.[7] The binding constant[43] for monoclonal antibody 8E11 and BPDE−16-mer is $\sim 2.5 \times 10^8\ M^{-1}$. We found that the presence of SSB protein (1.2 μM) in the mixture of anti-BPDE antibody (8 nM) and BPDE−16-mer (8 nM) did not enhance the complex formation. Instead, it decreased the complexes between the antibody and BPDE−oligonucleotide (data not shown). The strong nonspecific binding may result in steric hindrance and nonexposure of the epitope for antibody binding.

In summary, the use of nonspecific proteins, such as BSA and IgG, greatly enhanced the complex formation, increased the stability of antibody and complexes, and improved the reproducibility of analysis. The resulting improvements in detection limit and the reduction in the amounts of antibody needed for the assay were realized with both small oligonucleotide and genomic DNA as the target analytes. The strategy reported here can be applied to other immunoassays.

(47) Rykova, E. Yu.; Pautova, L. V.; Yakubov, L. A.; Karamyshev, V. N.; Vlassov, V. V. *FEBS Lett.* **1994**, *344*, 96−98.

ACKNOWLEDGMENT

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), National Cancer Institute of Canada, and the Canada Research Chairs Program. The authors thank Ms. Jane Lee of the Cross Cancer Institute for technical assistance on cell incubation and DNA extraction from A549 cells and Ms. S. Jessa for the experiments on single-stranded DNA binding.

Received for review October 6, 2002. Accepted November 7, 2002.

AC026204A