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Immobilization of DNA in Polyacrylamide Gel for the Manufacture of DNA and DNA–Oligonucleotide Microchips

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Activated DNA was immobilized in aldehyde-containing polyacrylamide gel for use in manufacturing the MAGIChip (microarrays of gel-immobilized compounds on a chip). First, abasic sites were generated in DNA by partial acidic depurination. Amino groups were then introduced into the abasic sites by reaction with ethylenediamine and reduction of the aldimine bonds formed. It was found that DNA could be fragmented at the site of amino group incorporation or preserved mostly unfragmented. In similar reactions, both amino–DNA and amino–oligonucleotides were attached through their amines to polyacrylamide gel derivatized with aldehyde groups. Single- and double-stranded DNA of 40 to 972 nucleotides or base pairs were immobilized on the gel pads to manufacture a DNA microchip. The microchip was hybridized with fluorescently labeled DNA-specific oligonucleotide probes. This procedure for immobilization of amino compounds was used to manufacture MAGIChips containing both DNA and oligonucleotides.

Key Words: **DNA; gel immobilization; DNA microchips.**

Immobilization of DNA on a solid surface through various nonspecific and ionic interactions and by chemical crosslinking (1–3) has found wide application in many fields. Immobilization of DNA on a nitrocellulose filter by baking, followed by DNA hybridization (3), is one of the most often used procedures in molecular biology. Genome mapping (4–6), DNA sequence analysis (7), and studies of gene expression (8, 9) in recent years have required large-scale production of DNA arrays on nitrocellulose and plastic sheets, glass surfaces, and other supports.

The MAGIChip (microarrays of gel-immobilized compounds on a chip) is being developed for various applications (10–13). MAGIChips contain a regular set of polyacrylamide gel pads ($60 \times 60 \times 20$ μm and larger) fixed on a glass slide and spaced from each other with a hydrophobic surface. Polyacrylamide gel provides a stable support with low fluorescent background. Compounds such as oligonucleotides, DNA, proteins, and antibodies have been chemically immobilized in the gel pads (12). A number of chemical and enzymatic reactions have been carried out in selected or all microchip gel pads (10, 12, 14). The gel has a capacity for three-dimensional immobilization 100 times and more compared with two-dimensional glass support. Higher concentration of immobilized compounds increases sensitivity of detection. MAGIChip can be stored for several months and hybridized 15–50 times.

DNA microchips can be used for the same purposes as DNA arrays. DNA immobilization was shown to be compatible with amino–oligonucleotide immobilization. The hybrid oligonucleotide and DNA microchips can combine the advantages of both oligonucleotide and DNA chips in their use for different applications. Hybrid microchips containing cDNA and oligonucleotides as expressed sequence tags may be proposed to identify and fractionate (14) expressed gene families that correspond to homologues and cross-hybridized cDNAs as well as individual genes within the families that differ by unique sequences. Oligonucleotide–DNA microchips may have essential advantages in searching for sequence-specific proteins and low-molecular-weight ligands and in studying thermodynamic param-

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eters of their complexes (A. V. Fotin, *et al.*, unpublished work).

Here a simple procedure for immobilizing DNA in polyacrylamide gel pads for the manufacture of DNA microchips will be described. The procedure involves partial depurination of DNA, reaction of DNA aldehyde groups with ethylenediamine to incorporate amino groups into DNA, and attachment of DNA through these amine groups to aldehyde-derivatized polyacrylamide gel (14). Before immobilization, DNA can either be partially and randomly fragmented or remain mostly unfragmented. DNA microchips were tested by hybridization with complementary fluorescently labeled oligonucleotide probes. DNA immobilization was shown to be compatible with amino–oligonucleotide immobilization on the same chip.

MATERIALS AND METHODS

Oligonucleotides were synthesized on an ABI-394 DNA–RNA synthesizer (Applied Biosystems, Foster City, CA). C7-Amine CPG, N-MMT-C6 aminomodifier, and deoxynucleoside phosphoramidites were purchased from Glen Research (Sterling, VA); *Taq* polymerase was from Promega (Madison, WI); and all other reagents were from Aldrich (Milwaukee, WI).

PCR Amplification, Depurination, and Aminomodification of DNA

Single-stranded (ss)[2] DNA 218 and 972 nt long (from positions 161 to 378 and 161 to 1132, respectively) and double-stranded (ds) DNA of 218 and 972 bp (also from positions 161 to 378 and 161 to 1132) were amplified from human carcinoma-associated antigen GA733-2 cDNA (clone GA733-2-2) (16) by conventional and asymmetric polymerase chain reaction (PCR) with primers 5′-GACTTTTGCCGCAGCTCAGGAAGAAT (forward), 5′-TGTTCTGGAGGGCCCCTTCAGGTTTT (reverse for 218-nt-long fragment), and 5′-TGTCTTCGTCCCACG-CACACACATTT (reverse for 972-nt-long fragment). A 227-bp DNA fragment from the human leukocyte-associated antigen (HLA) DQα 0201 gene (positions 32 to 258) (17) was amplified by conventional PCR with primers 5′- ATGGTGTAAACTTGTACCAGT (forward) and 5′-TTGGTAGCAGCGGTAGAGTTG (reverse). PCR amplification was carried out on a RoboCycler Gradient 96 temperature cycler (Stratagene, La Jolla, CA) according to the following protocol: 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 40 cycles. Preheating was carried out at 95°C for 5 min. PCR products were purified on QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol.

The synthetic 40-nt oligomer 5′-TACCAGTTTTACG-GTCCCTCTGGCCAGTACACCCATGAAT was depurinated for 3–60 min in 20 μl of 80% formic acid at 20°C, precipitated with 15 vol of 2% $LiClO_4$ in acetone, and dried (18). PCR-amplified DNA was depurinated for 3–60 min by the same procedure. Complete depurination of model oligonucleotide 5′-F-T_6GT_8 labeled by Texas Red (rhodamine 101 sulfochloride) at the 5′-end was performed by incubation in 20 μl of 80% formic acid at 60°C for 90 min, followed by precipitation of $LiClO_4$ in acetone (18).

Aminomodification of DNA with fragmentation. Ten nanomoles of fluorescently labeled, fully depurinated oligonucleotide 5′-F-NH-T_6dRT_8; 10 nmol of 40-mer depurinated for 3–60 min; or 100 pmol of PCR-amplified DNA depurinated for 3–60 min was incubated in 50 μl of 0.5 M ethylenediamine hydrochloride, pH 7.4, at 37°C for 3 h and then treated with 4 μl of freshly prepared 0.1 M $NaBH_4$ solution for 30 min. Four microliters of 20% ethylenediamine was added and DNA or oligonucleotides were precipitated with 1 ml of 2% $LiClO_4$ in acetone and dried.

Aminomodification of DNA without fragmentation. Depurinated oligonucleotides or DNA were dissolved in 50 μl of 20 mM solution of ethylenediamine hydrochloride in 100 mM sodium acetate buffer, pH 4.4. Four microliters of 0.5 M sodium cyanoborohydride in dry acetonitrile was immediately added; the reaction mixture was incubated at room temperature for 0.5 h and precipitated with 1 ml of 2% $LiClO_4$ in acetone, washed with acetone, and dried.

Some fragmented and unfragmented amino–oligonucleotides and amino–DNA were purified on C-18 columns (Alltech, Nicholasville, KY; 500 mg, high load) before immobilization. They were dissolved in 1 ml of 0.1 M triethylamine acetate solution (pH 7), applied to the columns, and washed consecutively with water, 5% acetic acid, water, and 2% aqueous $LiClO_4$, 5 ml each. Oligonucleotides were eluted from columns with 1.5 ml of 50% acetonitrile and evaporated *in vacuo.* The average yield of oligonucleotides after purification was 50%. Purification did not significantly affect the immobilization and hybridization patterns of DNA microchips.

Immobilization of Aminomodified DNA and Amino–Oligonucleotides on a Gel Micromatrix

A micromatrix containing 100 × 100 × 20-μm polyacrylamide gel pads fixed on a glass slide and spaced 200 μm from each other was manufactured by photopolymerization (12). A 4% solution of acrylamide–bisacrylamide mixture (19:1), containing 0.35% (mol) of *N*-(5,6-di-*O*-isopropyidene)hexylacrylamide (14) in 0.1 M sodium phosphate (pH 7) in 50% glycerol was used.

[2] Abbreviations used: dR, 2-deoxyribose residue; ds (ss), double (single) strand; HLA, human leukocyte-associated antigen; ON, oligonucleotide(s).

The micromatrix was washed with 50 ml of water, placed for 5 min in 50 ml of 2% trifluoroacetic acid, washed with 50 ml of water, dried, and placed for 30 min in 50 ml of freshly prepared 0.1 M $NaIO_4$. The micromatrix was then washed with 500 ml of water, dried, and used for DNA immobilization.

Solutions of amino–oligonucleotides (0.3 mM) or amino–DNA (0.03 mM) in distilled water were applied with a manual loading device to micromatrix gel pads (1 nl to each pad) containing aldehyde groups (12, 14). Loaded micromatrices were placed in 100 ml of 0.1 M solution of borane–pyridine complex in water-saturated chloroform to reduce the Schiff base. Twenty milliliters of water was loaded above the chloroform layer; matrices were incubated under a two-phase system for 12 h and washed with ethanol, then water, and dried. Microchips with attached oligonucleotides or DNA were treated for 20 min in 0.1 M $NaBH_4$ to reduce residual gel aldehydes. Finally, the DNA microchips were washed for 3 h in 50 ml of water and dried.

Fluorescent Labeling of Oligonucleotide Probes

Up to 25 nmol of a synthetic oligonucleotide containing the 3′-terminal amino group was dissolved in 70 μl of 100 mM aqueous $NaHCO_3$–acetonitrile (2:1) solution. Freshly prepared solution of 50–100 μg of Texas Red (19) in 30 μl of dry acetonitrile was added and the mixture was allowed to react at 0° for 4 h. The excess of fluorescent label was extracted four times with 0.5 ml of water-saturated butanol. Oligonucleotide was then precipitated with 2% $LiClO_4$ in acetone, purified by gel electrophoresis, and used for hybridization with DNA microchip.

Hybridization of Oligonucleotides with Immobilized DNA

One hundred picomoles of oligonucleotide 5′-CATGGGTGTA-F (ON1), 5′-CAGAGGGACC-F (ON2), 5′-AAAACTGGTA-F (ON3), 5′-GTACTGGCC-F (ON4), 5′-GATGAAGGCA-F (ON5, positions 311–320 in GA733-2-2 DNA fragment), 5′-TGGTGATAGCAGTTG-F (ON6, positions 946–960 in GA733-2-2 DNA fragment), or 5′-AGTCTCCTTCTCCAGGTCCACATAGAACTC-CTCGTCT-F (ON7, positions 93–129 in HLA DQα DNA fragment) were dissolved in 100 μl of 6× SSPE buffer (Sigma) with 1% Tween 20, loaded on the microchip containing immobilized DNA, and incubated at 25°C (ON1–ON5), 35°C (ON6), or 45°C (ON7) for 16 h. Fluorescence image analysis of hybridized microchips was carried out with a multicolor fluorescence microscope equipped with a charge-coupled device camera (10).

RESULTS

Depurination and Incorporation of Amino Groups into DNA

Figure 1 shows the reactions of coupling between ethylenediamine and depurinated DNA with or without fragmentation. These reactions are followed by crosslinking of aminomodified DNA with aldehyde-functionalized polyacrylamide gel.

Oligonucleotide depurination and coupling of depurinated oligonucleotide with ethylenediamine under various conditions were analyzed with fluorescently labeled synthetic 15-mer 5′-F-T_6GT_8. The reaction products were analyzed quantitatively by gel electrophoresis and gel scanning (Fig. 2). Depurinated 5′-F-T_6-dR-T_8 oligomer (band *B*, lane 2) showed electrophoretic mobility very close to that of the unmodified oligonucleotide (band *A*, lane 1). Depurination caused minor fragmentation (about 10%), yielding fast-moving hexanucleotide 5′-F-T_6-p (band *E*) as a product of δ-elimination reaction (18, 20), and formation of insignificant amount of unidentified degradation products (minor bands in lane 2 in the region of band *C*). Incubation of the depurinated oligonucleotide with ethylenediamine for 3 h at 37°C and pH 7.4 produced β-elimination and strand cleavage at the depurinated site with about 70% yield. Reduction of the generated fragment with $NaBH_4$ produced amino–oligonucleotides 5′-F-T_6-dR-NH-C_2H_4-NH_2 with increased electrophoretic mobility (band *C*, lane 3). About 15% of full-length aminomodified oligonucleotide (band *D*, lane 3) was also observed.

Coupling of the depurinated oligonucleotide with ethylenediamine at pH 4.4 in the presence of sodium cyanoborohydrate as a reducing agent prevented fragmentation and promoted the formation of full-length amino–oligonucleotide 5′-F-T_6-dR(-NH-C_2H_4-NH_2)T_8, with a yield of about 90% (band *D* in Fig. 2, lane 4). The amino–oligonucleotide was contaminated with less than 5% of the initial depurinated oligonucleotides (band *B*) and with the δ-elimination product (band *E*).

Immobilization and Hybridization of 40-nt Synthetic DNA

Synthetic 40-mer oligonucleotide depurinated under various conditions was coupled with ethylenediamine with and without fragmentation. Aminomodified 40-mer was immobilized within the 100 × 100 × 20-μm aldehyde-functionalized gel pads of the micromatrix. Schiff bases in amino–40-mer/gel aldehyde adducts were reduced with pyridinium–borane complex in a three-layer system consisting of (i) gel pads, (ii) 0.1 M Py · BH_3 complex in water-saturated chloroform, and (iii) water. This technique was used to supply water and the reducing agent to each gel pad from chloro-

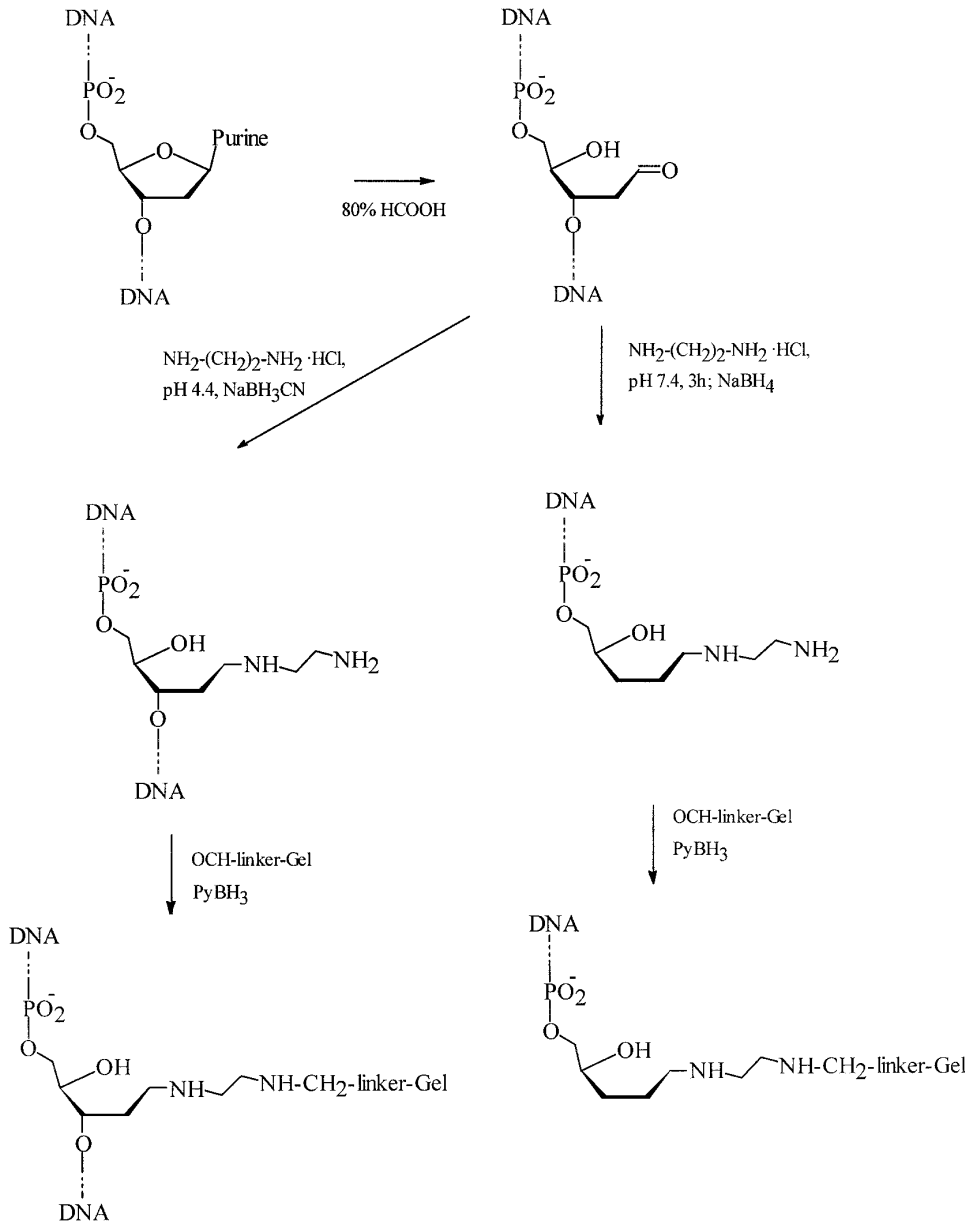

FIG. 1. Chemical modification and immobilization of DNA on gel support.

form; the chloroform prevents cross-contamination between neighboring gel pads by DNA solutions.

The efficiency of DNA immobilization was monitored by hybridization of the microchip with four fluorescently labeled oligonucleotides, ON1–ON4. The pattern of hybridization of the DNA microchip with ON3 is shown in Fig. 3A. Hybridization was more efficient for higher levels of depurination (40 and 60 min of acid treatment) of both fragmented and nonfragmented DNA. Extended acidic treatment increases the level of depurination, reduces DNA length, and enhances DNA immobilization and, as a result, the efficiency of hybridization. However, excessive depurination should interfere with hybridization because fewer purine bases are pairing. The hybridization signals of ON3 with 40-mer immobilized at higher levels of depurination were comparable with fluorescence intensity of a control gel pad of the microchip containing immobilized fluorescently labeled amino–oligonucleotide. The labeled oligonucleotide and 40-mer were loaded at the same concentrations, indicating that control oligonucleotide and DNA have similar immobilization efficiencies.

Quantitative data on the hybridization of four probes with immobilized 40-mer depurinated to different levels are shown in Figs. 3B and 3C. As expected, pyrim-

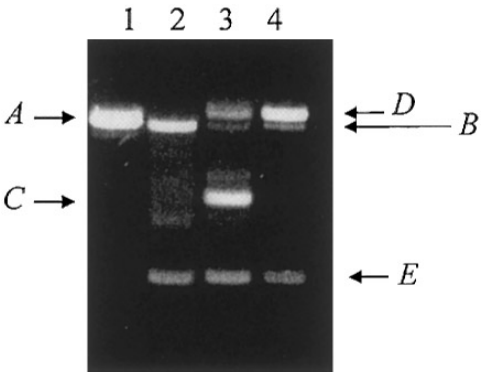

FIG. 2. Depurination and incorporation of amino group into oligonucleotide. Gel electrophoresis of fluorescently labeled oligonucleotide 5′-F-NH-T_6GT_8 before (lane 1) and after (lane 2) depurination, followed by incubation with 0.5 M ethylenediamine hydrochloride, pH 7.4, for 3 h and reduction with $NaBH_4$ (lane 3) or treated with sodium cyanoborohydride in 20 mM ethylenediamine in 0.1 M sodium acetate buffer, pH 4.4 (lane 4). Band *A*, unmodified oligonucleotide. Band *B*, depurinated oligonucleotide. Band *C*, aminomodified fragment 5′-F-T_6-dR-NH-C_2H_4-NH_2. Band *D*, full-length amino–oligonucleotide. Band *E*, product of δ-elimination.

idine-rich oligonucleotides ON1 (60% purines) and ON4 (44% purines), which bind to purine-rich sites in immobilized depurinated 40-mers, showed weaker hybridization signals when compared with purine-rich ON2 (70% purines) and ON3 (70% purines). This difference is lower with 40-nt DNA immobilized without fragmentation (Fig. 3C). Apparently, the hybridization is less sensitive to the presence of an excised base than to a chain break induced by depurination in the DNA duplex. On the other hand, less efficient hybridization of ON1 and ON4 may be related to their addressing to the 3′-end of the target immobilized sequence. The aminomodification and chain break result in immobilization of the fragment positioned at the 5′ but not at the 3′ part of the DNA sequence relative to the scission point. Therefore, the probes complementary to the 5′ region of the DNA fragmented at several points provide higher hybridization signals than probes complementary to the 3′-terminal regions.

Immobilization and Hybridization of Long DNA

Overlapping ss and ds of 218- and 972-nt-long fragments of human carcinoma-associated antigen GA733-2 cDNA (clone GA733-2-2) and a 227-nt-long fragment of the HLA DQα 0201 gene were prepared by conventional and asymmetric DNA amplification. They were depurinated, treated with ethylenediamine, and immobilized within the gel pads of the micromatrix. Untreated DNA was used as control.

Figure 4 shows the hybridization of the DNA microchip with fluorescently labeled oligonucleotides ON5 (10 nt long), ON6 (15 nt long), and ON7 (37 nt long) complementary to DNA having 972 and 218 (ON5), 972 (ON6), and 227 (ON7) bp. All of the oligonucleotide probes showed specific binding to complementary immobilized DNA. Gel elements that were loaded with a control sample of untreated DNA (lane 8 in Fig. 4), as well as empty gel pads (not shown), did not give noticeable hybridization signals. Increasing the time of depurination from 3 to 30 min resulted in growth of fluorescence; however, longer acidic treatment (60 min) did not enhance hybridization of either ss or ds DNAs (Fig. 4). As indicated by the hybridization patterns, fragmented DNA immobilized more evenly inside the gel pads, while nonfragmented DNA, in particular 972-nt- or bp-long DNA, was bound mostly to the surface (Figs. 4A and 4B). Immobilized ss and ds DNA of different sizes showed similar hybridization patterns, indicating that two complementary strands of immobilized DNA do not interfere with each other during hybridization with short oligonucleotides.

DISCUSSION

Partial acidic depurination of DNA is a simple reaction that generates active aldehyde groups at abasic sites (21–23). It is used in the Maxam–Gilbert sequencing procedure (24, 25), for attaching fluorescence labels to DNA (18), and for immobilization of DNA on hydrazide-activated polyacrylamide gel (12). DNA depurination can be carried out under mild conditions at 37°C and neutral pH after partial depurination with dimethylsulfate. This reaction has been used to map proteins (24) and to sequence the arrangement of proteins along DNA in their complexes (26).

A procedure for aminomodification and fluorescent labeling of DNA was recently developed (18). The amino groups were introduced by reacting the aldehyde groups of depurinated DNA with ethylenediamine, followed by reduction. A similar procedure based on reductive coupling is used here for DNA immobilization in aldehyde-derivatized polyacrylamide gel (Fig. 1). The reaction of gel aldehyde groups with amino–oligonucleotides was effective for immobilizing oligonucleotides on modified polyacrylamide gel to manufacture gel microchips (14). In this procedure, the aldehyde-containing gel interacts with 3′- or 5′-amino oligonucleotides to produce aldimine bonds, which are further reduced into stable secondary amines. The coupling is carried out under mild conditions with a high (60–90%) yield, providing highly stable linkage between the gel and the immobilized oligonucleotides.

Aminomodified DNA is immobilized on aldehyde-containing polyacrylamide gel through the same course of reactions: formation of aldimine bonds and their reduction with borane–pyridine complex. Aldehyde groups in the gel are produced by treatment of polyacrylamide gel containing 1,2-diol groups with $NaIO_4$

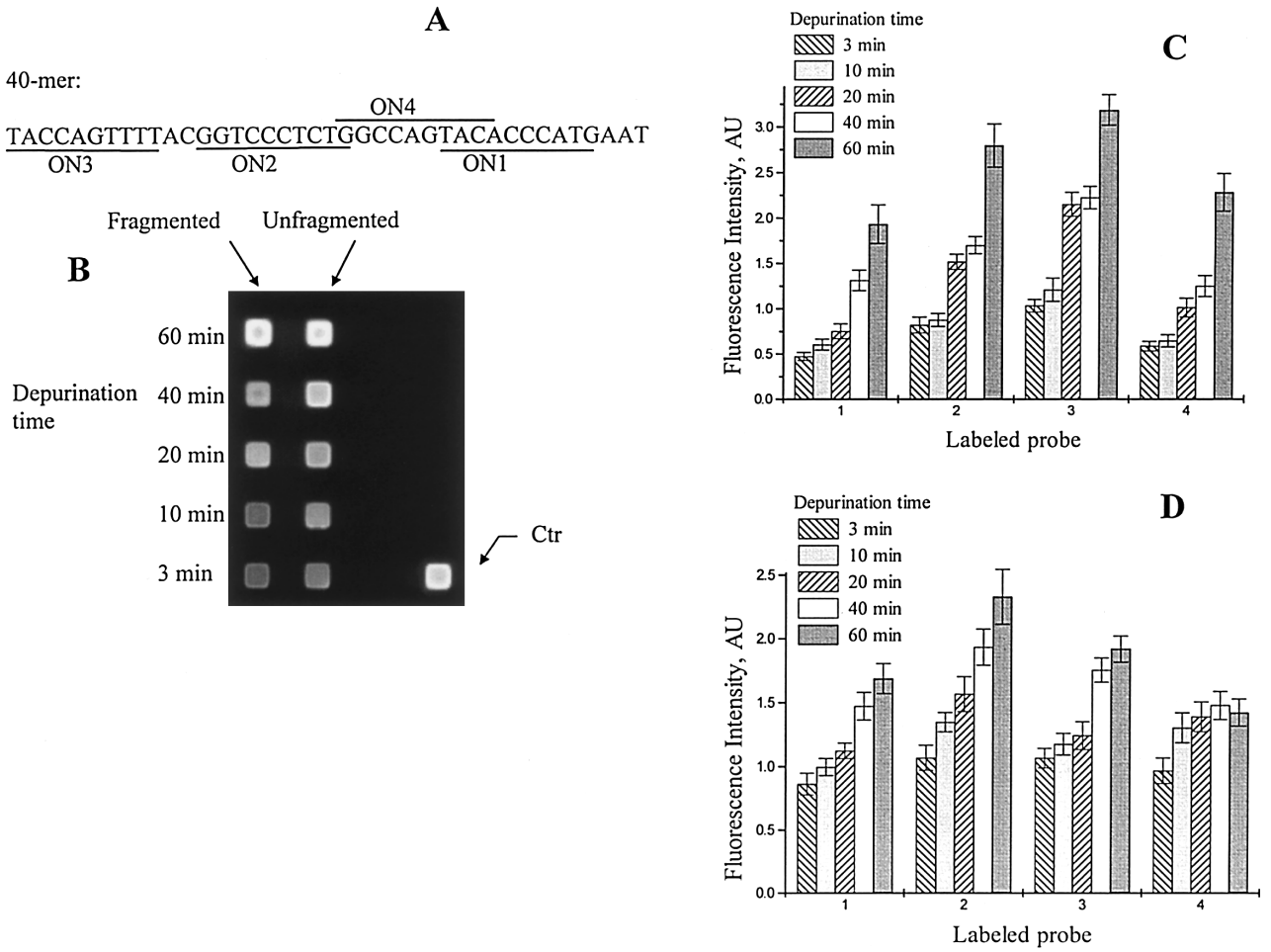

FIG. 3. Hybridization of oligonucleotides with microchip-immobilized 40-mer. (**A**) Structure of 40-nt synthetic DNA. Sequences in 40-mer that are complementary to ON1–ON4 are underlined. (**B**) Pattern of hybridization of immobilized fragmented and unfragmented 40-mer with fluorescently labeled ON3. Ctr–control labeled 8-mer. (**C** and **D**) Quantitative analysis (means ±SE for three repeats) of the hybridization of four fluorescently labeled oligonucleotides, ON1–ON4, with microchip-immobilized fragmented (**C**) and unfragmented (**D**) 40-mer depurinated for different times (min). Fluorescence intesities are given in arbitrary units.

(14). During acidic depurination, DNA is fragmented at up to about 10% of depurinated sites as the result of δ-elimination reactions. The coupling of ethylenediamine with aldehyde groups of depurinated DNA causes Schiff bases to form, which undergo β-elimination at pH 7.4, yielding short 3′-aminomodified fragments. However, essentially no DNA fragmentation was observed when ethylenediamine modification was carried out at lower pH 4.4 in the presence of sodium cyanoborohydride. The latter was added for immediate reduction of the formed Schiff bases.

The low porosity of the polyacrylamide gel restricts the size of immobilized DNA to 150–200 bases and proteins up to 170,000 Da (12). (Longer DNA and larger proteins may be attached to the highly developed surface of the gel.) In addition, ss DNA has a tendency to form hairpins, which interfere with its hybridization. For these reasons, it is more advantageous in some cases to use controlled fragmentation of DNA for manufacturing of DNA microchips. Fragmentation of DNA to pieces of 50–150 nucleotides should not significantly affect its hybridization efficiency and selectivity. Using more porous gels can facilitate the immobilization of longer DNA (A. D. Mirzabekov, *et al.*, unpublished work).

Immobilization performed under the conditions of this study yielded similar patterns of hybridization for both ss and ds DNA. Both DNA strands seemed to be attached to the gel separately and their possible interaction did not interfere with hybridization. Amino–DNA and amino–oligonucleotides are also immobilized under the same conditions. Therefore, this method can be used to manufacture mixed oligonucleotide–DNA microchips, which may have advantages for monitor-

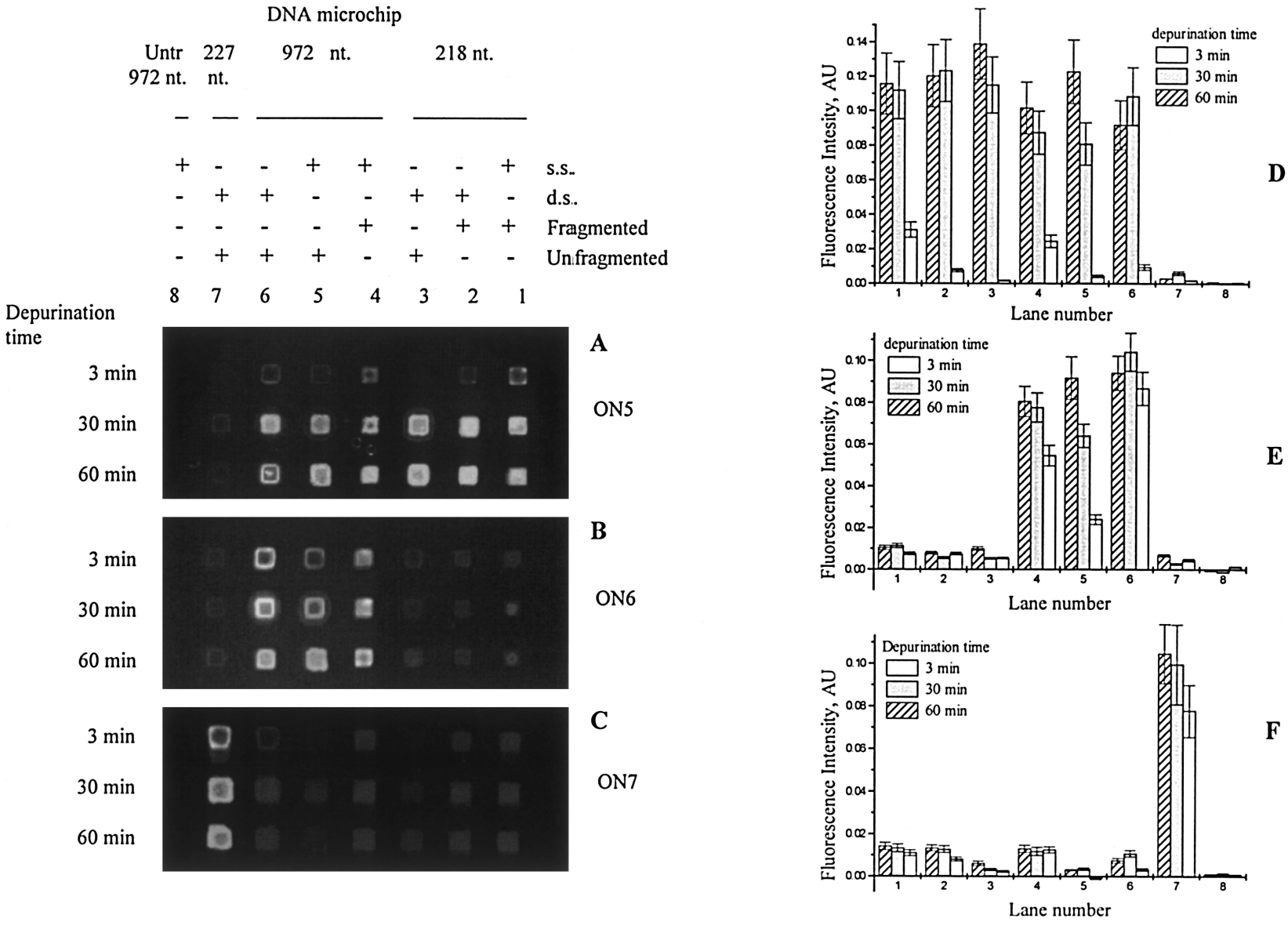

FIG. 4. Hybridization of microchip-immobilized fragmented and unfragmented ss and ds DNA. Partially overlapping PCR-amplified DNA of 218 (lanes 1–3) and 972 (lanes 4–6) nt from human-carcinoma-associated antigen GA733-2 (clone GA733-2-2), along with 227-nt-long DNA from the HLA DQα 0201 gene (lane 7), were immobilized on the microchip. The DNA was then hybridized with labeled oligonucleotides: (**A**) ON5 (10-mer, complementary to 218- and 972-nt-long DNA), (**B**) ON6 (15-mer, complementary to 972-nt-long DNA), and (**C**) ON7 (complementary to 227-bp DNA). Control lane 8 corresponds to 972-bp DNA that was applied to the microchip gel pads without depurination or ethylenediamine treatment. (**D, E,** and **F**) Quantitative analysis (means $\pm$SE for three repeats) of the hybridization of labeled oligonucleotides ON5 (**D**), ON6 (**E**), and ON7 (**F**) with DNA microchip. Fluorescence intensities are given in arbitrary units.

ing gene expression by hybridization with cell-extracted RNA or their cDNA copies. The same procedure can be used for immobilization of proteins (12) and other aminofunctionalized biomolecules to manufacture MAGIChips.

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