Hybridization of DNA Targets to Glass-Tethered Oligonucleotide Probes

*Wanda G. Beattie, Lin Meng, Saralinda L. Turner, Rajender S. Varma, Dat D. Dao, and Kenneth L. Beattie**

Abstract

Hybridization of nucleic acids to surface-tethered oligonucleotide probes has numerous potential applications in genome mapping and DNA sequence analysis. In this article, we describe a simple standard protocol for routine preparation of terminal amine-derivatized 9-mer oligonucleotide arrays on ordinary microscope slides and hybridization conditions with DNA target strands of up to several hundred bases in length with good discrimination against mismatches. Additional linker arms separating the glass surface from the probe sequence are not necessary. The technique described here offers a powerful tool for the detection of specific genetic mutations.

Index Entries: Genosensors; sequencing by hybridization; oligonucleotide arrays; DNA chips; genome mapping; DNA sequencing; DNA probes; mutation detection.

1. Introduction

Hybridization of nucleic acids to surface-tethered oligonucleotide probes is being developed in several laboratories *(1–7)* and has numerous potential applications in genome mapping and sequence analysis *(8–14)*. Anticipated uses of oligonucleotide arrays include sequencing by hybridization, mutation detection, species identification, DNA marker analysis, individual identification, and profiling of gene expression. Although oligonucleotide array hybridization is conceptually straightforward and many important applications of the technology can be envisioned, numerous technical parameters must be understood before oligonucleotide array hybridization can be implemented for routine laboratory use. These parameters include:

1. Chemical and physical properties of the support surface;
2. Composition and length of a chemical linker between the support surface and the oligonucleotide probe;
3. Density of attachment of probes to the surface;
4. Length and strandedness (single- vs double-) of target DNA molecules;
5. Sequence and length of probes; and
6. Hybridization and washing conditions.

A variety of solid surfaces may be suitable for oligonucleotide array hybridization, and for each support material a variety of chemical linkages between surface and probe may suffice. This article reports the results of hybridization experiments performed using oligonucleotide probes tethered to glass slides via epoxysilane-amine covalent linkage. A simple standard protocol is reported for routine preparation of oligonucleotide arrays on ordinary microscope slides, and conditions are given that enable specific hybridization of PCR targets to glass-tethered probes, with minimal nonspecific binding of target molecules to the surface.

2. Materials and Methods

2.1. Oligonucleotide Synthesis

Oligonucleotides were synthesized at Genosys Biotechnologies (The Woodlands, TX), using

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standard phosphoramidite chemistry *(15)* and a segmented synthesis strategy that enables simultaneous synthesis of large numbers of oligonucleotides *(16–18)*. Phosphoramidites for introduction of 5'- or 3'-amino modifiers and triethylene glycol spacers into oligonucleotides were obtained from Glen Research (Sterling, VA).

2.2. Glass Slide Preparation

Glass microscope slides were prepared for probe attachment by soaking in 1N HNO_3 for 30–60 min followed by a rinse in H_2O and sonication in hexane, acetone, and absolute ethanol for 10 min each. The slides were then dried and soaked for 5 h at 80°C in a solution composed of 24 parts anhydrous xylene, 8 parts 3-glycidoxypropyltrimethoxysilane and 1 part *N,N*-diisopropylethylamine *(19)*. Excess reagents were removed by three washes in tetrahydrofuran and the slides were dried and stored desiccated under vacuum until the oligonucleotide probes were attached.

2.3. Oligonucleotide Probe Attachment

Oligonucleotide probes were synthesized with either a 3'- or 5'-terminal amine modification and dissolved in H_2O. An aliquot (typically 200 nL) of each probe, diluted to the desired concentration (1–100 µM) in water, was applied to the epoxysilanized glass slide with a Hamilton Microlab 2200 workstation (Reno, NV) equipped with a multiprobe head. In the absence of an automated workstation, the glass slides can be placed on top of a template and the probes can be applied by hand with a micropipet. The slides were placed in a prewarmed high-humidity chamber consisting of a small sealable plastic box containing a shelf for holding the slides, a water reservoir at the bottom, and filter paper at the top. After incubation at the desired temperature and time, slides were washed in H_2O at 60°C, 10 m*M* triethylamine, pH 9.2, at room temperature, twice more with hot H_2O, then stored dry at room temperature.

2.4. Preparation of Target DNA

Oligonucleotide target DNA was 5'-end labeled by action of polynucleotide kinase in the presence of [γ-^{32}P]ATP or [γ-^{33}P]ATP. The labeled oligonucleotide was loaded onto a Microcon-3 microconcentrator (Amicon, Beverly, MA) and washed three times with H_2O to remove unincorporated [γ-^{32}P] ATP. To prepare natural single-stranded target DNA by the polymerase chain reaction (PCR), the PCR reaction was conducted with one of the primers labeled with biotin at the 5'-end and the other primer labeled with ^{32}P. The PCR product was processed with a Millipore (Bedford, MA) Ultrafree spin-filter (30,000 mol-wt cutoff) to remove excess PCR primers, then the retained material was applied to an AffiniTip *Strep 20* column (Genosys Biotechnologies). The single-stranded eluate from the column was desalted and concentrated by ethanol precipitation.

2.5. Hybridization Conditions

Hybridizations were performed in a solution containing 3.3*M* tetramethylammonium chloride (TMACl), 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.1% SDS, and 10% polyethylene glycol-8000 (PEG). Target DNA was dissolved in the hybridization solution at a concentration of 15–30 fmol/µL (PCR products) or 300–500 fmol/µL (oligonucleotide targets) and a minimum of 2000 cpm/µL. A 20-µL aliquot of the target solution was applied to the area of the slide containing the array of attached probes, covered with a cover slip and incubated at the desired temperature and time. When the oligonucleotide array occupied the entire slide, a 60-µL aliquot of probe was applied, and covered with a 24 × 50-mm cover slip. After incubation for the desired time, cover s lips were removed and the slides were washed a minimum of 1 h in a solution of 3.3*M* TMACl, 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, and 0.1% SDS at 22°C. The results of the hybridization were obtained by phosphorimager analysis, using a Fuji BAS 1000 Bio-Imaging Analysis System (Stamford, CT).

3. Results

3.1. Attachment of Oligonucleotides to Glass

The general strategy that we have used to tether oligonucleotides to glass surfaces is illustrated in Fig. 1. The glass surface is first activated by treatment with epoxysilane reagent as detailed in Materials and Methods. The oligonucleotide probe

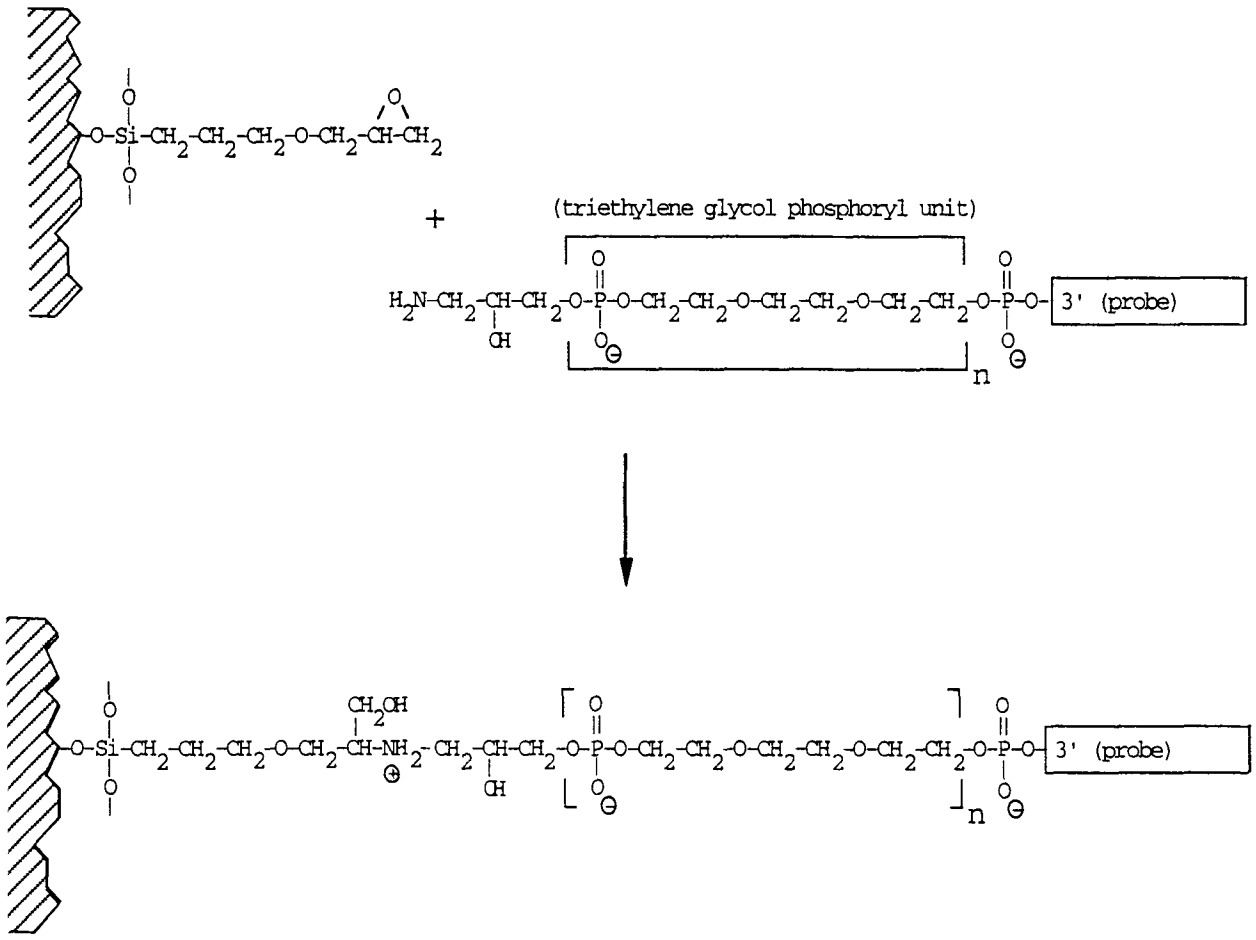

Fig. 1. Chemistry of probe attachment to glass (SiO_2) surface. 3'-amino modified oligonucleotide probes, bearing multiple triethylene glycol phosphoryl units, react with epoxysilane monolayer to form a secondary amine linkage.

is synthesized to contain a terminal amine linker at either 5'- or 3'-terminus. In Fig. 1, the oligonucleotide is shown to contain a 3'-amino modification, plus an optional triethylene glycol phosphoryl spacer, which can be introduced during the chemical synthesis to provide a variable length spacer arm between the glass surface and the oligonucleotide sequence. This spacer was used to assess whether placement of the oligonucleotide further from the support surface would improve the hybridization efficiency. At the bottom of Fig. 1 is shown the expected chemical structure of the covalent linkage resulting from specific reaction between 3'-terminal amine and epoxysilanized glass.

Several solution conditions have been compared for oligonucleotide attachment. As reported previously *(6)*, attachment under alkaline conditions (100 m*M* KOH) provided good specificity for attachment of oligonucleotides containing terminal primary amines (compared with unmodified probes). Figure 2 shows a comparison of attachment under the following conditions: (1) water; (2) 5% glycerol in water; (3) 100 m*M* KOH; and (4) 10 m*M* KOH. The phosphorimager display on the left was obtained after application and drying of 1-µL aliquots of a 10 µ*M* solution of 3'-amino modified 9-mer in each of the above solutions (no washing). The image on the right was obtained after attachment and washing (*see* Materials and Methods). Although a similar extent of attachment occurred under the four conditions (approx 1% [1 nmol] of applied probe), the attachment in water gave a sharply confined spot of attached probe, while in base and glycerol the

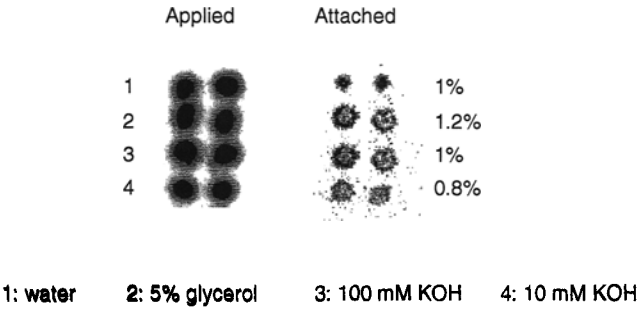

Fig. 2. Comparison of probe attachment in various solutions. 3'-amino-modified 9-mer probes (1 μL aliquots of a 10-μ*M* solution) in (1) water, < 2) 5% glycerol, (3) 100 m*M* KOH, and (4) 10 m*M* KOH were attached to a glass slide using conditions described in the methods section. Left: phosphorimager display of probe applied, Right: phosphorimager display of probe attached and the extent of attachment (%).

probe solution tended to spread out during attachment. Based upon these data, together with the observation (discussed later) that attachment in basic conditions increases the nonspecific target DNA binding during the hybridization step, we recommend attachment in water.

3.2. Hybridization of Oligonucleotide Targets to Glass-Tethered Probes

It is relevant to know whether hybridization of target DNA strands to an array of surface-tethered probes is optimal at a particular surface density of immobilized probes. For example, will hybridization be hindered if the probes are spaced too densely on the surface, possibly because of steric crowding and/or excessive charge density? To address this general issue, we applied 200-nL aliquots of 5'-^{32}P-labeled 9-mer probes, with and without 3'-amino derivatization, to epoxysilanized microscope slides, at various temperatures (20, 37, and 65°C) and concentrations (0.5–100 μ*M*), then estimated the attachment density by phosphorimager analysis after reaction for various times (0.5, 2, and 22 h). The ability of identical unlabeled probe arrays (prepared in parallel) to hybridize with labeled target strands was then assessed. A [5'-^{32}P]21-mer, bearing a perfect complement to the arrayed 9-mer probe, was dissolved at 443 fmol/μL in hybridization buffer and 60 μL were applied to

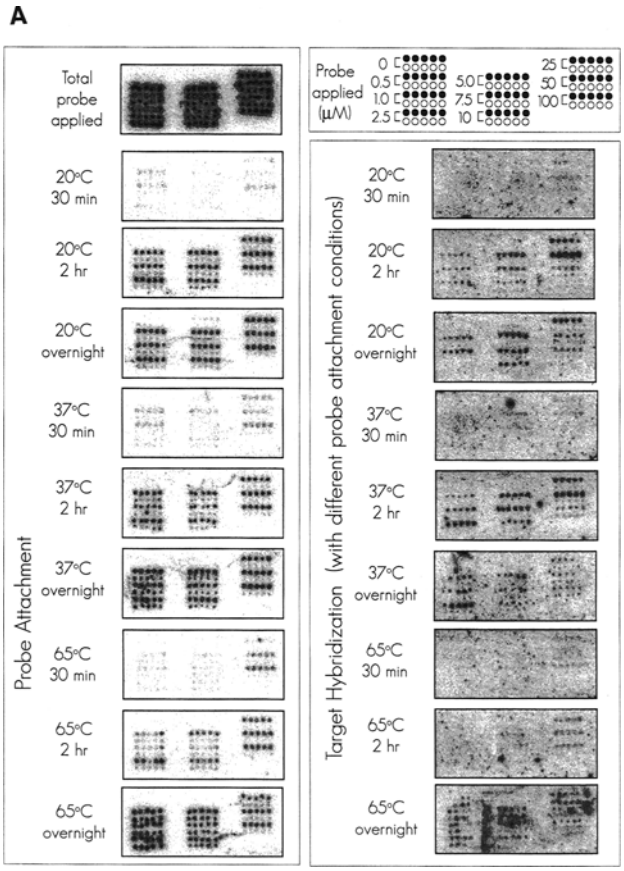

Fig. 3. (A) Influence of attachment conditions on the extent of probe attachment and on the extent of target DNA hybridization. 5'-^{32}P-labeled 9-mer probes (200-nL aliquots), with and without 3'-amino derivatization, were applied to epoxysilanized microscope slides, at various temperatures (20, 37, and 65°C), times (0.5, 2, and 22 h), and concentrations (0.5–100 μ*M*). Left, phosphorimager display of probe applied (top) and probes attached. Right, template of probes attached to glass slides using various concentrations of probes with (closed circles) and without (opened circles) 3'-amino derivatization (top) and phosphorimager display of hybridization results.

each slide, covered with a cover slip, and incubated overnight at 6°C, then washed as described in Materials and Methods. A phosphorimager output from this experiment is shown in Fig. 3A. Attachment results are displayed on the left and hybridization results are shown on the right. The key in the lower right indicates the positioning of probes on the slide (in quintuplicate, bold circles

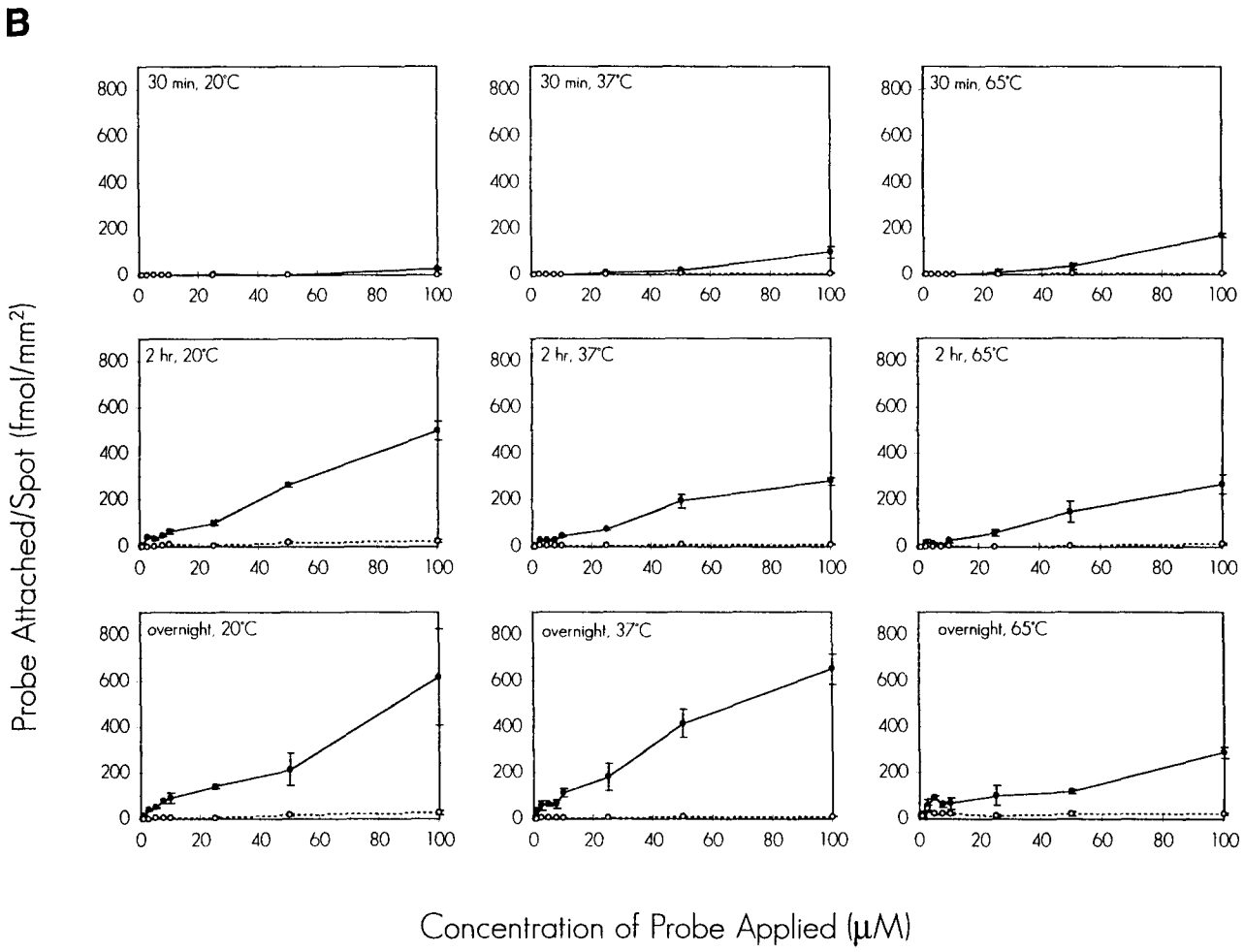

Fig. 3. *(continued on next page)* **(B)** The extent of attachment at different probe concentrations. The amounts of probe attached in fmol/mm^2 were plotted against the amounts of probe applied in μ*M* using various conditions described in the methods section.

for 3'-amino modified probes and thin circles for nonamino probes).

The extent of probe attachment at different probe concentrations is plotted in Fig. 3B. The greatly enhanced attachment of 3'-amino modified vs nonamino probes (also evident from the phosphorimager data of Fig. 3A) indicates that covalent attachment of probes to the activated glass occurred preferentially via the terminal primary amine function. Except at the shortest time-point, probe attachment occurred to greater extent at 20 and at 37°C than at 65°C. The extent of covalent attachment increased with probe concentration and time of incubation, although relatively little further attachment occurred after 2 h of incubation.

From the quantity of 3'-amino, 5'-^{32}P-labeled oligonucleotides attached (typically 50–500 fmol probe over a 1.5-mm diameter spot), and assuming that the effective surface area of the glass is about twice that of an atomically smooth surface, we calculate that the epoxysilane-amine attachment reaction yields a surface density of oligonucleotide probes on the glass slide in the range, 10^{10}–10^{11} probes/mm^2, which equates to a molecular spacing of 30–100 Å between adjacent surface-tethered probes within each region of attachment on the glass slide.

The extent of hybridization of complementary sequence-bearing 21-mer target strands to the 9-mer probe arrays is tabulated according to reaction

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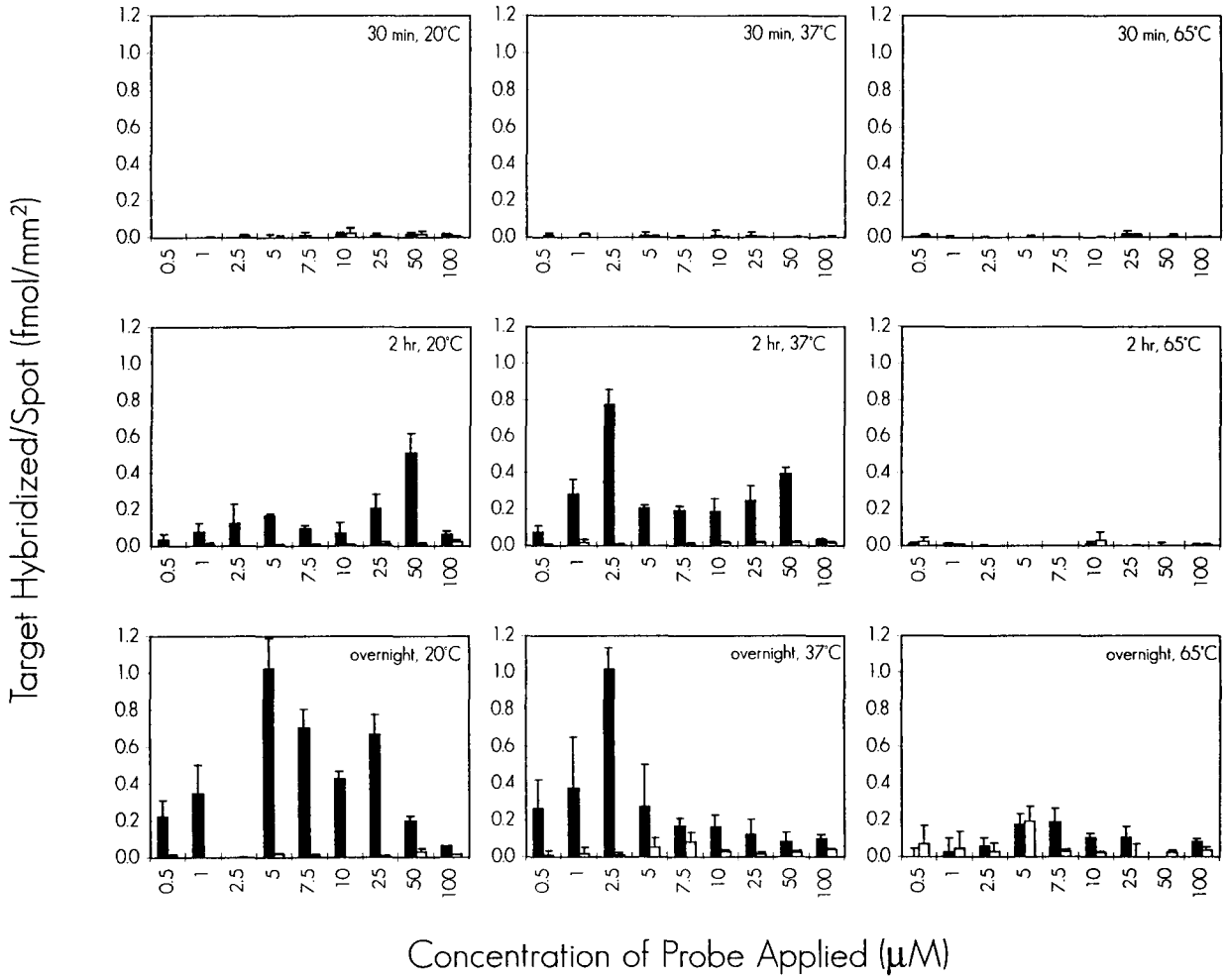

Fig. 3. *(continued from previous page)* **(C)** Tabulation of the extent of hybridization according to reaction time, temperature, and probe concentration.

time, temperature, and probe concentration in Fig. 3C. Very little hybridization occurred after 30 min of attachment at all temperatures and probe concentrations. In this experiment, the hybridization was relatively poor when attachment was conducted at 65°C, in comparison with attachment at 37 and 20°C, although we have achieved acceptable hybridization following 65°C attachment in other experiments. This variability possibly reflects difficulty in controlling the drying of small droplets at high temperature.

The extent of hybridization as a function of probe attachment density is plotted in Fig. 3D, for attachment at 20°C overnight and 37°C for 2 h (conditions yielding the greatest hybridization). To compensate for a larger area of attachment at highest probe concentrations, attachment density was calculated as fmol/mm^2. These data indicate that attachment at 5 μ*M* probe concentration is optimal for hybridization, and that control of probe density on the surface will be important for optimizing the hybridization.

Another vital concern in hybridization experiments involving oligonucleotide arrays is the need to minimize nonspecific binding of ^{32}P label (from the kinase reaction) to the surface. We found that,

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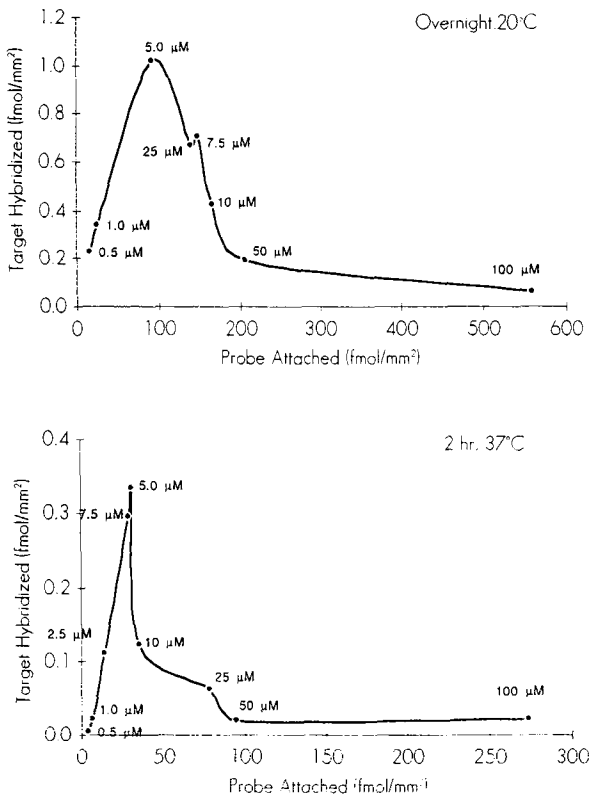

Fig. 3. **(D)** The extent of hybridization as a function of probe attachment density. Numerical values on the curves within the figures represent the concentration of probe applied.

in the absence of any prehybridization "blocking agent," a high background of nonspecific binding of ^{32}P label (owing to residual [γ-^{32}P]ATP and/or 5'-labeled target strands) to the glass slide occurs. We therefore surveyed the effectiveness of a number of prehybridization agents commonly used in membrane hybridizations (as well as some other small molecules that have functional groups similar to ATP), in reducing the nonspecific background. Figure 4 displays some typical results obtained in this study. Each rectangle represents the phosphorimager data obtained after hybridization of a ^{32}P-labeled 22-mer target strand to an array of 9-mer and 12-mer surface-tethered probes, among which a few specific hybridization spots were expected to be seen (the sequence details are not relevant here and are thus omitted). In the absence of any prehybridization step (slide pictured at the bottom), there was a relatively high level of background radioactivity. Minimal nonspecific background was seen when a prehybridization step was carried out using 2.5 mg/mL dried milk, 10 m*M* ATP, 10 m*M* tripolyphosphate (TPP), and 2.5 mg/mL tryptone. The other agents gave little or no reduction in nonspecific binding of ^{32}P. The fact that ATP and tripolyphosphate were effective, but ribose, adenine, and adenosine were ineffective suggests that the nonspecific binding involves electrostatic interactions (possibly of residual [γ-^{32}P]ATP), which are effectively competed out by the triphosphate moiety. Because of the convenience, low cost, and high water solubility of tripolyphosphate, we now include soaking of slides in 10 m*M* TPP as a standard prehybridization step. For reasons not yet understood, the prehybridization step (with 10 m*M* TPP, 10 m*M* ATP, or 0.25% dried milk) is also necessary to enable washing away of bound target molecules (with hot water), which permits the oligonucleotide array to be repeatedly used in hybridization experiments (data not shown).

To examine whether the length of the linker arm separating the oligonucleotide probe from the glass surface influences the efficiency of hybridization, we utilized a series of 3'-amino modified 9-mer probes containing from 0–10 additional triethylene glycol phosphoryl residues separating the oligonucleotide sequence and the 3'-amine function. The relative separation of the 9-mer sequence from the glass surface (in numbers of rotatable bonds), depicted in Fig. 5, ranged from about 1/4 the length of the 9-mer to over twice the length of 9-mer in this series. The rationale for this experiment is that tethering of the oligonucleotide too c lose to the surface might restrict access of target strands to the probe sequence during hybridization, either because of simple steric hindrance or because of a "Stern" layer of solvent at the surface, within which diffusion of molecules is known to be relatively slow compared to diffusion in bulk solution.

A 9-mer probe of sequence AGTGGAGGT, containing the series of variable linker arms as described, was attached to the glass slide in the

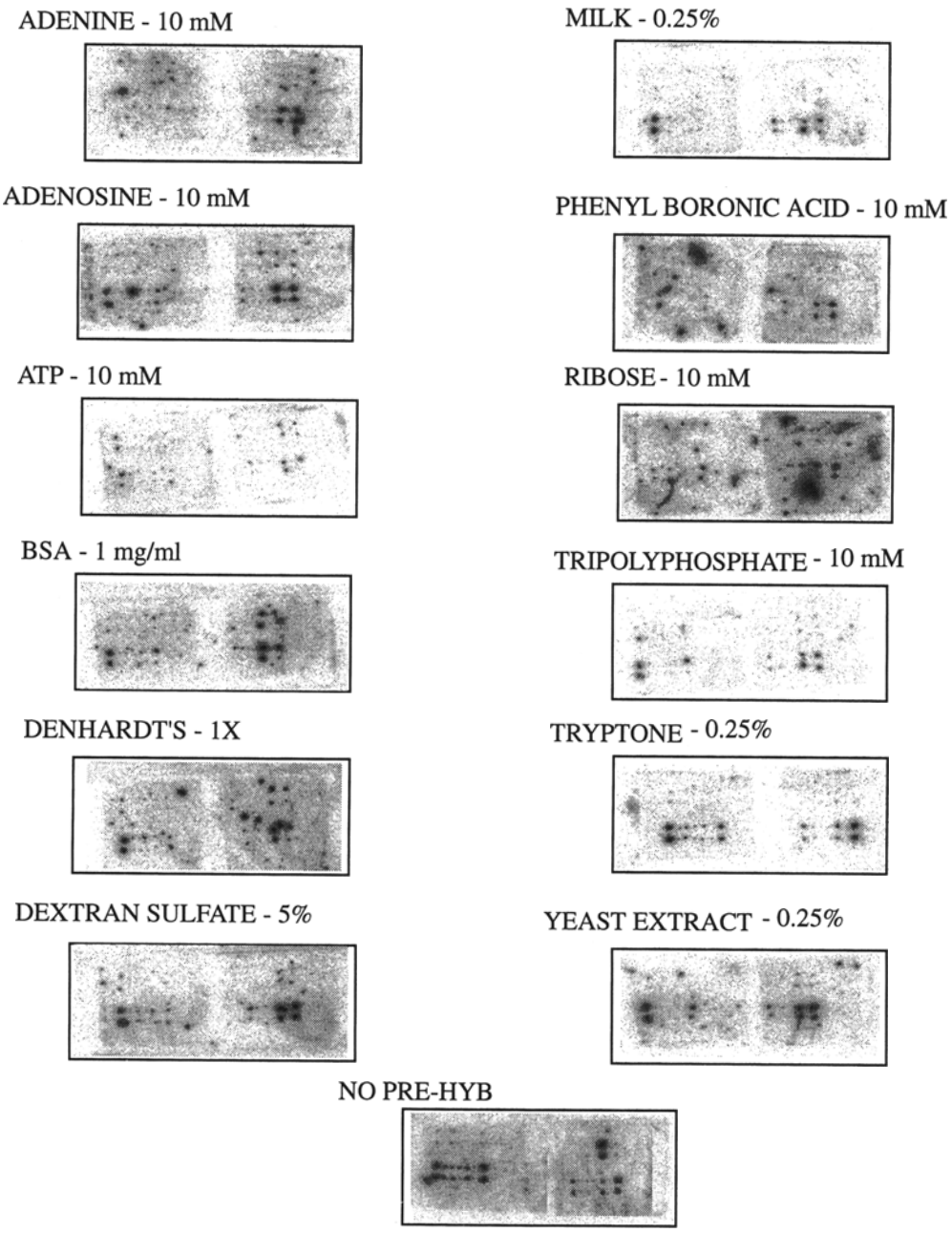

Fig. 4. Influence of various prehybridization treatments on nonspecific binding of ^{32}P during hybridization of labeled 21-mer with array of 9-mer probes. The array of 9-mers was designed to produce a few specific hybridization spots (representing perfect matches with the target) whereas the majority of probes contained one or more mismatches with the target. Prior to hybridization, the slides were soaked at room temperature for 1 h in the "blocking solution" containing the additives indicated above each slide image.

pattern indicated at the bottom left of Fig. 6, in three groupings, then hybridized with three different 21-mer target strands, depicted in Fig. 6B. Only the middle (wild-type) target was perfectly complementary to the 9-mer probe sequence, whereas the upper (549 mutation) and lower (551 mutation) target molecules (bearing base substitutions found in the CFTR gene) both formed penultimate mismatches when paired with the wild-type probe. The hybridization results shown

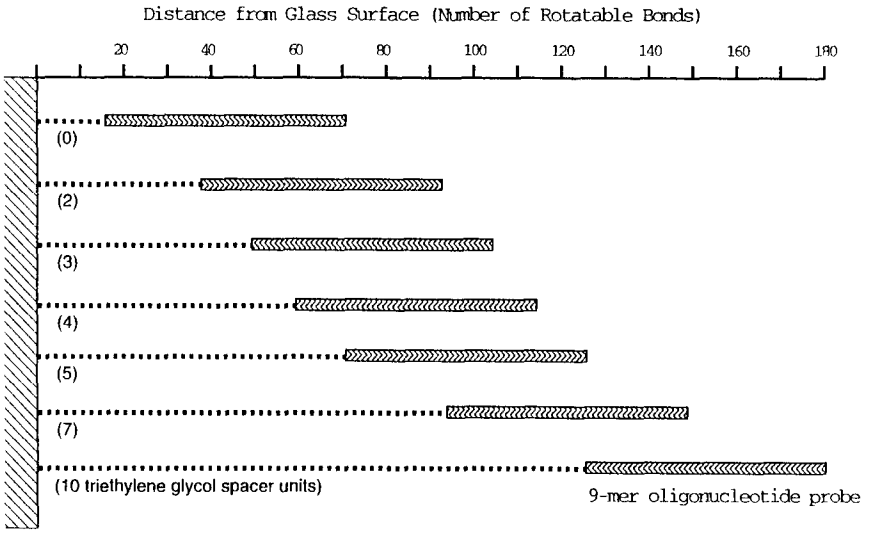

Fig. 5. Probes linked to glass via variable spacer arms. The distance (number of rotatable bonds) of 3'-amino 9-mer probes containing 0–10 additional triethylene glycol phosphoryl residues from glass surface.

in Fig. 6C indicate that with short synthetic target strands there is no appreciable effect of linker arm on hybridization intensity. Furthermore, there was no apparent influence of added linker arm on the mismatch discrimination in these target-probe combinations.

The results of a similar experiment involving terminal mismatches between 9-mer probe without linker arm and mutant 21-mer targets are shown in Fig. 6D. Although mismatch discrimination was less efficient with the terminal mismatches than the penultimate mismatches, as expected from solution studies *(20)*, there was still acceptable discrimination, even against a relatively stable terminal G·T mismatch in this target-probe hybridization.

3.3. Hybridization of PCR Fragments to Surface-Bound Oligonucleotides

To be a useful analytical tool, oligonucleotide array hybridization must function with natural nucleic acid analytes, most commonly fragments produced with the PCR. Thus, we asked whether the procedures defined above for hybridization of oligonucleotide targets would function with PCR fragments of various lengths. In addition, we investigated whether the addition of spacer arms would be needed for hybridization of longer strands to the oligonucleotide array. The series of linker arms used in this experiment are depicted in Table 1. In this experiment, we included triethylene glycol phosphoryl linkers as well as linkers composed of T residues interspersed with triethylene glycol residues. Each triethylene glycol spacer is approximately equivalent in length to two T residues. The results of the hybridization experiment with complementary 9-mer probes and various length PCR product target strands, shown in Fig. 7, indicate that glass-tethered 9-mers are capable of capturing target strands as long as 1300 bases (the longest fragments tested), although the hybridization signals were reduced at longer target length. Although some improvement in hybridization of the longest targets may be achieved by providing increased spacer between the glass and the oligonucleotide sequence, this effect may be offset by the significant added cost associated with incorporating linker residues into the probes during the chemical synthesis. The data shown in Fig. 7 also demonstrate that the inclusion of 10% polyethylene glycol (PEG) to the hybridization reaction significantly improves the hybridization signal in the case of PCR fragments of length greater than about 200 bases.

Finally, the data of Fig. 8 demonstrate that mismatch discrimination can be achieved in the "real

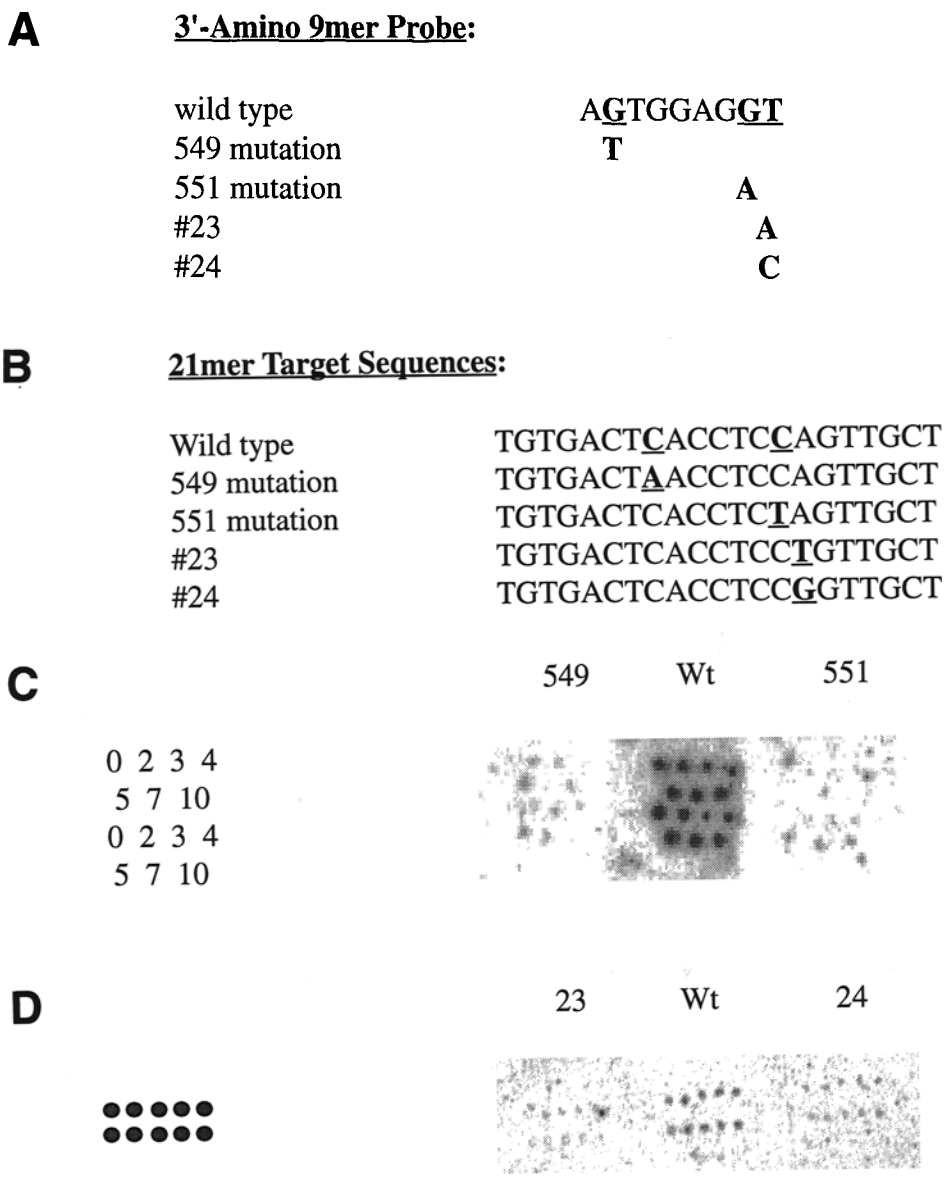

Fig. 6. Mutation detection of the CFTR gene. **(A)** Sequence of the 9-mer wild-type (wt) and mutant probes of the cystic fibrosis transmembrane receptor gene used in this study. **(B)** Sequence of 21-mer targets. **(C)** Number of triethylene glycol phosphoryl residues linked to 9-mer probes (left) and the hybridization results of the 549, wt, and 551 targets to the wt probe (right). **(D)** Oligonucleotide array (left) and hybridization results of the target #23, wt, and #24 to the wt probe (right). Spacer units were not used in this study.

world" hybridization of a PCR fragment with a surface-tethered 9-mer probe. The wild-type, 549 mutant, and 551 mutant 9-mer probes (containing no additional linker arm in this case) were tethered to the glass in three rows of quintuplicate spots (15 spots for four different groupings), then hybridized with wild-type, 549 mutant, and 551 mutant 21-mer targets, as well as with wild-type 282-base (single-stranded) PCR fragment. Although the labeling of PCR fragment was less intense than that of the short synthetic targets, there was clearly good discrimination against the penultimate mismatches on the 9-mer probe, with the "549" G·A mismatch selected against more efficiently

Table 1
Oligonucleotide Probes with Variable Length Terminal Spacers

3'-NH_2 Probes	
0	AGTGGAGGT-GLASS
2	AGTGGAGGT-l-l-GLASS
4	AGTGGAGGT-l-l-l-l-GLASS
5	AGTGGAGGT-l-l-l-l-l-GLASS
7	AGTGGAGGT-l-l-l-l-l-l-l-GLASS
5'-NH_2 Probes	
11	GLASS-l-l-TTTTTTl-l-AGTGGAGGT
12	GLASS-TTTTl-l-TTTTl-l-AGTGGAGGT
13	GLASS-TTTTl-TTTTl-AGTGGAGGT
15	GLASS-TTTTTTl-l-TTTTTTl-l-AGTGGAGGT
16	GLASS-TTTTTTl-TTTTTTl-AGTGGAGGT
17	GLASS-TTTTTl-TTTTTl-AGTGGAGGT
20	GLASS-TTTTTl-TTTTTl-TTTTTl-AGTGGAGGT

l = triethylene glycol phosphoryl residue.

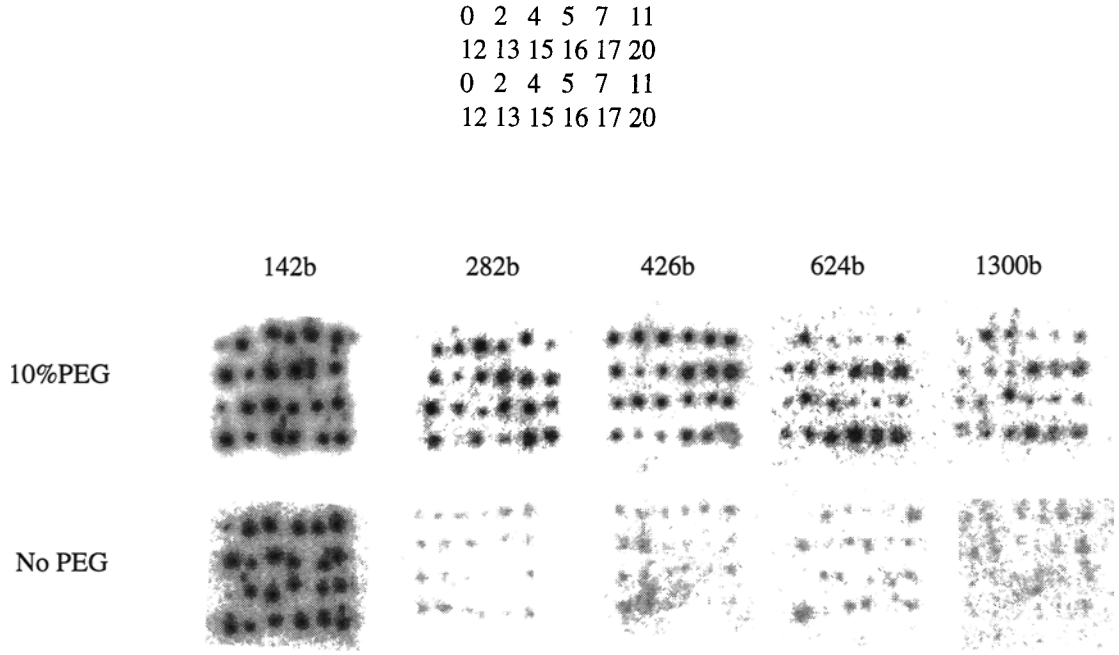

Fig. 7. Necessity for spacer arms and the effect of polyethylene glycol (PEG) on hybridization. Oligonucleotide probes specific for the wt of the CFTR gene were attached to glass slides using a variety of spacer units. The numbers in the top panel correspond to the type of spacer units described in Table 1. Hybridization was carried out as described in the methods section using ^{32}P-labeled target sequence of various lengths (142, 282, 426, 624, and 1300 b PCR products), with or without PEG.

than the "551" G·T mismatch. These results are encouraging in anticipation of the use of oligonucleotide arrays to detect mutations in PCR fragments.

4. Discussion

In this paper we have reported conditions for covalent attachment of terminal amine-derivatized oligonucleotides to ordinary microscope

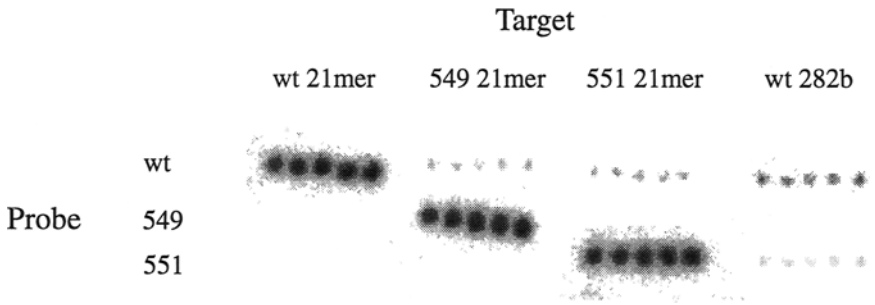

Fig. 8. Mismatch discrimination of the CFTR gene mutation using PCR fragment. The wild-type, 549 mutant, and 551 mutant probes (containing no additional linker arm in this case) were tethered to the glass in three rows of quintuplicate spots (15 spots for four different groupings), then hybridized with wild-type, 549 mutant, and 551 mutant 21-mer targets, as well as with wild-type 282-base (single-stranded) PCR fragment. Hybridization was carried out at 6°C overnight in 3.3*M* TMACl, 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.1% SDS, 10% PEG using 200 fmol/μL targets.

slides, and have demonstrated the ability of glass-tethered 9-mer probes to hybridize with DNA target strands of up to several hundred bases in length, with good discrimination against mismatches. In addition, conditions are described that minimize nonspecific target DNA binding to the glass, and our results have shown that with the possible exception of long (>1 kb) target strands, expensive additional linker arms separating the glass surface from the probe sequence are not necessary. The protocols reported here will enable any laboratory to prepare and use oligonucleotide hybridization arrays for a variety of sequence analyses experiments, such as mutation detection. The synthetic oligonucleotide probes remain the major expense of this type of work (an array of 100 9-mer probes may typically cost $2000–$3000). However, a standard 0.2-μ*M* synthesis will supply a sufficient probe for inclusion of an oligonucleotide in many thousands of hybridization arrays. Although advanced genosensor technology involving miniaturized DNA chips and sophisticated detection systems is under development in several research groups, the basic technology described here can immediately be applied as a research tool in the ordinary laboratory, utilizing existing methods for oligonucleotide synthesis, fluid delivery, and hybridization detection. Phosphorimager detection of hybridized ^{32}P, ^{33}P, or ^{35}S-labeled PCR fragments typically requires a few minutes, and inexpensive X-ray film detection (requiring a few hours) is sufficient. For applications that require greatest sensitivity or nonradioactive tags, chemiluminescent detection of hybridization within the oligonucleotide array may be performed using commercially available kits.

Use of oligonucleotide arrays for a specific task (e.g., detection of mutations in a specific genetic region) may require adjustment of conditions, such as probe length and hybridization conditions. Nevertheless, the protocols reported here provide reasonable guidelines for experiments utilizing oligonucleotide arrays in sequence analysis, and the cumulative experience of researchers will bring technical improvements and new applications of this new technology.

Acknowledgments

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