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Genetics

DNA analysis and diagnostics on oligonucleotide microchips

(sequencing by hybridization/hybridization pattern)

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ABSTRACT **We present a further development in the technology of sequencing by hybridization to oligonucleotide microchips (SHOM) and its application to diagnostics for genetic diseases. A robot has been constructed to manufacture sequencing "microchips." The microchip is an array of oligonucleotides immobilized into gel elements fixed on a glass plate. Hybridization of the microchip with fluorescently labeled DNA was monitored in real time simultaneously for all microchip elements with a two-wavelength fluorescent microscope equipped with a charge-coupled device camera. SHOM has been used to detect β-thalassemia mutations in patients by hybridizing PCR-amplified DNA with the microchips. A contiguous stacking hybridization technique has been applied for the detection of mutations; it can simplify medical diagnostics and enhance its reliability. The use of multicolor monitoring of contiguous stacking hybridization is suggested for large-scale diagnostics and gene polymorphism studies. Other applications of the SHOM technology are discussed.**

The hybridization of filter-immobilized DNA with oligonucleotides has been designed for the identification of mutations (1, 2). However, this approach is too cumbersome for screening of many mutations—e.g., in the case of β-thalassemia, the number of mutations exceeds 100 (3). The hybridization of DNA with a large set of short oligonucleotides has been suggested for DNA sequencing (4–9). The use of an array of surface-immobilized oligonucleotides opens a way for large-scale screening of mutations and studies of gene polymorphism. Several approaches have been suggested for preparing such arrays: oligonucleotide synthesis on a glass surface (8–10) or immobilization of presynthesized oligonucleotides on a surface (11–13) and into a microchip three-dimensional gel element (5, 14). We refer to the latter technique as the sequencing by hybridization to oligonucleotide microchip (SHOM) method. It appears that SHOM will be a promising method for *de novo* DNA sequencing after some technical problems are solved (15).

Here we report an advance in the development of SHOM technology and SHOM application for sequence analysis and diagnostics. A robot has been constructed for manufacturing microchips. Oligonucleotides are applied by the robot and immobilized into microchip gel elements fixed on a glass plate. A chip that is 1 × 1 cm may contain 20,000–30,000 40 × 40 μm elements. Fluorescently labeled DNA is hybridized on the microchip. The signals from DNA samples labeled with different fluorophores are analyzed by a specially designed two-wave length microscope equipped with a charge-coupled device (CCD) camera. The gel support increases the capacity for oligonucleotide immobilization and facilitates hybridization and mismatch discrimination (14–16). However, it applies a restriction on the size of the DNA that can diffuse into the gel. Fragmentation, or the use of short DNA pieces, allows us to avoid this restriction as well as to decrease the interference of hairpin structures on hybridization with oligonucleotides.

SHOM has been applied to diagnostics for genetic diseases. A number of β-thalassemia mutations were reliably (in a "yes or no" way) identified by the SHOM method. The identification of DNA base changes in the course of the diagnostics was simplified by using a contiguous stacking hybridization (CSH) approach (5, 14–17). CSH was developed to enhance the efficiency of SHOM. For example, CSH of DNA with an 8-mer microchip in the presence of 5-mers gives rise to a rather stable contiguous 13-bp duplex; a mismatch in the 5-mer part of the duplex prevents its formation. Such CSH can increase the length of DNA to be sequenced from 200 up to 4000 bases; CSH of 5-mers with a microchip of 65,536 octamers simulates the use of the microchip holding 67,108,864 13-mers (17).

The results obtained demonstrate the efficiency of SHOM applications for DNA sequence comparison and diagnostics for genetic diseases.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides were synthesized with a 394 DNA/RNA synthesizer (Applied Biosystems). Oligonucleotides fluorescently labeled at the 5′ terminus were synthesized by using FAM (6-carboxyfluorescein) and HEX (hexachloninated analogue of FAM) amidites (Applied Biosystems). The synthesis of oligonucleotides for immobilization started with 3-methyluridine, located at the 3′ end (14). Oligonucleotides and DNA for hybridization were also labeled at the 3′ end as follows: 100 pmol of an oligonucleotide were incubated at 37°C for 1 h in 20 μl of reaction mixture containing 100 mM cacodilate buffer, pH 6.8/1 mM $CaCl_2$/0.1 mM DTT/200 pmol of tetramethylrhodamine (TMR)-conjugated dUTP/10 units of terminal deoxinucleotidyl transferase (Promega) (18). Another procedure for labeling oligonucleotide containing the 5′ amino group was carried out with an excess of *N*-hydroxysuccinimide ester of 5-carboxy TMR (Molecular Probes) in dimethyl sulfoxide (Aldrich) with 50 mM sodium borate buffer (pH 9.0) at 60°C for 30 min. The

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Abbreviations: SHOM, sequencing by hybridization to oligonucleotide microchips; CSH, contiguous stacking hybridization; TMR, tetramethylrhodamine; ss, single stranded; ds, double stranded; CCD, charge-coupled device; FAM, 6-carboxyfluorescein; HEX, hexachloninated analogue of FAM; IVS, intervening sequence.

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oligonucleotides were purified on NAP-5 columns prepacked with Sephadex G-25 (Pharmacia).

PCR. PCR amplifications of DNA from patients were performed by an adapted procedure (19) with some modifications. All reaction were carried out in a thermocycler PHC-1 (Techne Laboratories, Princeton) in 100 μl containing 40 mM KCl, 20 mM Mops (pH 8.0), 2 mM $MgCl_2$, 100 mM each dATP, dCTP, dGTP, dTTP, 0.3 mM each amplification primers, and 2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus). Initially, PCR amplification of a 421-bp-long fragment was carried out with 1 ng of genomic DNA and the primers 5′-TGCCAGAAGAGCCAAGGACAGG-3′ and 5′-TAAGGGTGGGAAAATAGACC-3′. The reaction conditions were 30 cycles of 40 s at 93°C, 30 s at 67°C, and 30 s at 72°C. Five microliters of the PCR were transferred to another reaction mixture for amplification with nested primers. Nested primers, 5′-CATTTGCTTCTGACACAACT-3′and5′-TCTCCTTAAACCTGTCTTG-3′, were used to amplify 176-bp-long DNA fragment for 25 cycles (30 s at 90°C, 30 s at 50°C, 20 s at 72°C). A 176-bp PCR product was purified by a QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. About 5 μl of the 176-bp PCR product were used next for the nested PCR amplification of 32 bp DNA with 5′ TMR-labeled primer 5′-CCCTGGGCAG-3′ and unlabeled 5′-GTCTTGTAACCTTG-3′ that was carried out for 25 cycles (30 s at 80°C, 30 s at 35°C). The 32-bp PCR product was purified by gel electrophoresis.

Single-Stranded (ss) DNA Preparation. ssDNA was prepared by single primer reamplification according to ref. 20 with some modifications. Some 100 ng of the double-stranded (ds) 176-bp fragment per 100 μl of PCR was used for amplification with fluorescently labeled primers: 5′-HEX-CTGTGGGGCAAGGTGAACG-3′ for an 85-base-long fragment and 5′-HEX-TGAAGTTGGTGGTGAGGCCC-3′ for a 62-base-long transient (25 cycles, 30 s at 90°C, 30 s at 50°C, 20 s at 72°C). Some 50 ng of the ds 32-bp fragment per 100 μl PCR was used for single primer reamplification with 5′-TMR-labeled primer: 5′-CCCTGGGCAG-3′ (25 cycles, 30 s at 80°C, 30 s at 35°C). Single-stranded amplification products were purified by electrophoresis in 8% polyacrylamide gel. The fragment was cut out and eluted in 1 M $LiClO_4$ and precipitated in 10 volumes of 2% $LiClO_4$ in acetone.

DNA Fragmentation. DNA was fragmented by chemical reactions used in the Maxam–Gilbert sequencing method. A 176-bp-long PCR DNA (3 μg) was dissolved in 20 μl of 80% formic acid (Merck) and incubated at room temperature for 10 min. The reaction was stopped by adding 20 volumes of cold 2% $LiClO_4$ in acetone and cooling for 10 min at −20°C. DNA was precipitated by spinning down in microcentrifuge for 3 min. The pellet was washed twice with acetone, air dryed and dissolved in 20 μl of 90% hydrazine hydrate (Sigma), incubated at room temperature for 30 min, and precipitated with 20 volumes of cold 2% $LiClO_4$ in acetone. DNA was then dissolved in 100 μl of 1 M piperidine and incubated at 95°C for 30 min followed by chloroform extraction and precipitation in 2% $LiClO_4$ in acetone. The resulting products were dephosphorylated twice using 1 unit of shrimp alkaline phosphatase in 10 μl of provided buffer (United States Biochemical) for 1 h at 37°C. Phosphatase was inactivated by heating at 95°C for 15 min. Fragments were labeled with 1 nmol of TMR-conjugated dUTP using terminal deoxinucleotidyl transferase as described above.

Microchip Manufacturing. A matrix of glass-immobilized gel elements was prepared as described (14) by polymerization of 20-μm thin polyacrylamide (8% acrylamide/0.28% bisacrylamide) gel on a glass surface treated by Bind-Silane (LKB). Strips of the gel were removed in *x*–*y* directions with a scribing machine (14), forming the array of the gel elements of the size 40 × 40 μm or 100 × 100 μm and spaced by 80 or 200 μm, respectively. The polyacrylamide gel was activated by substitusion of some amide groups with hydrazide groups by hydrazine–hydrate treatment. The glass space between the gel elements was hydrophobized by treatment with Repel-Silane (LKB). Oligodeoxynucleotides for immobilization were synthesised with 3′-terminal 3-methyluridine, activated by oxidizing with $NaIO_4$ to produce dialdehyde groups for coupling with the hydrazide groups of the gel (14). The solution of activated oligonucleotide was transfered on the micromatrix element with a specially devised one-pin robot (Fig. 1) (21). The reproducibility of the transfer is ±8%. The pin temperature is kept at a dew point to prevent drop evaporation or water condensation on the pin. After the transfer, the matrix temperature is decreased and water is condensed on the gel. The fully swollen gel matrix is covered by oil (Nujol mineral oil;

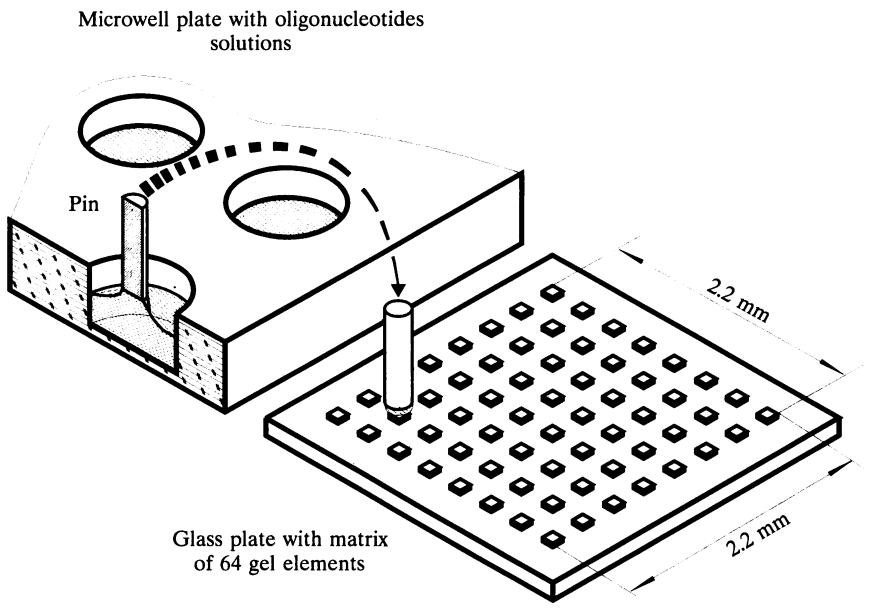

FIG. 1. The scheme of a one-pin robot for manufacturing oligonucleotide microchips. The 100-μm-thick pin has a hydrophobic side surface and a hydrophilic bottom surface. The pin transfers about 1 nl of an activated oligonucleotide solution from the well of the microtiter plate onto a microchip gel element. The pin temperature and its *x*–*y*–*z* movements are controlled by a computer.

Schering–Plough) and kept at 20°C for 48 h for quantitative oligonucleotide immobilization; then the oil was washed out by ethanol and distilled water. The microchips were dried and can be kept at 4°C for 1 year before use.

Hybridization, Washing, and Staining Procedures. All procedures were performed on a Peltier thermotable (working range from −5.0°C to +60.0°C). Hybridization of a microchip with fluorescently labeled DNA (1–5 pmol) or oligonucleotides (5 pmol) was carried out at 5°C for 30 min in 2–5 μl of the hybridization buffer (1 M NaCl/1 mM EDTA/1% Tween 20/5 mM sodium phosphate, pH 7.0) for oligonucleotides and in 0.1× buffer for DNA. Then, the microchip was washed with 100 μl of the hybridization buffer at 5°C for 10 s and covered with 5 μl of the same buffer. For ethidium bromide (EtdBr) staining, 1 mM solution of unlabeled DNA was hybridized with the microchip and stained with 1 mM EtdBr in 0.1× buffer for 5 min at 5°C.

Contiguous stacking hybridization was carried out in two steps. After initial hybridization of the microchip with PCR-amplified ssDNA as described above, the microchip was hybridized with 1 μl of a mixture of pentamers (5 pmol of each) in 1× buffer for 5 min at 4°C. The hybridized 5-mers were washed off the microchip at 20°C for 2 min in 1× buffer and the hybridizations were repeated under the same conditions with the other mixtures of 5-mers.

Image Analysis System. To monitor fluorescence signals, from either FAM or TMR and HEX, a two-wavelength epifluorescence microscope was designed in collaboration with the State Optical Institute (St. Petersburg, Russia). It includes 350-W high-pressure mercury lamp, Ploem opaque (22) with interference excitation and barrier filters, special optics, and a CCD camera (TE/CCD512SF; Princeton Instruments, Trenton, NJ). The ×3 objective with a 0.4 numerical aperture allows illumination of an object field up to 7 mm in diameter. It projects a 2.7 × 2.7-mm area of the microchip on the 8.1 × 8.1-mm matrix of a Peltier cooled CCD camera. The exposure time varied from 0.4 to 30 s, with a readout time of about 1.3 s. Variations in the sensitivity within the object area did not exceed 5%. The system allows work with a ×1.7 objective and the same aperture for analyzing a 5 × 5-mm microchip area. Two sets of filters for FAM or HEX and TMR (Omega Optical, Brattleboro, VT) can be quickly changed for analysis of these fluorophores. A four-color microscope is under construction. The image of the microchip on the CCD camera was displayed and analyzed on a PC using specially developed software. For printing, we used a linear transformation that brought the highest pixel values to the same level for all images. For digital estimation, the image of the microchip element was fully covered by a "square" (total signal, *S*) twice the size of the element. Then a frame (total signal, *F*) having an area equal to that of the square was constructed around the square. The signal of the element (E) was calculated as $E = S - F$.

RESULTS AND DISCUSSION

Microchip Manufacturing and Analysis. Polyacrylamide gel provides a maximal capacity of 50 mM for three-dimensional immobilization. However, much lower concentration (1.5 μM to 1.5 mM of oligonucleotides immobilized through a hydrazide bond) has been used. This corresponds to a range of 0.5–50 and 3–300 fmol of immobilized oligonucleotides per microchip element of the sizes 40 × 40 × 20 μm and 100 × 100 × 20 μm, respectively. That is more than 100 times higher than the two-dimensional immobilization capacity of a glass surface (14, 23). The high hybridization capacity of the gel enabled us to detect duplexes on the microchips by using also staining with ethidium bromide (not shown).

The gel support offers some other essential advantages. Under our conditions of immobilization, more than 70% of oligonucleotides can participate in hybridization and they are well spaced from each other. This prevents interference between different molecules of oligonucleotides and DNA during hybridization and enhances the efficiency of discrimination of perfect duplexes from mismatched ones. The effective temperature stability of the duplexes formed with gel-immobilized oligonucleotides depends on their concentration. This depen-

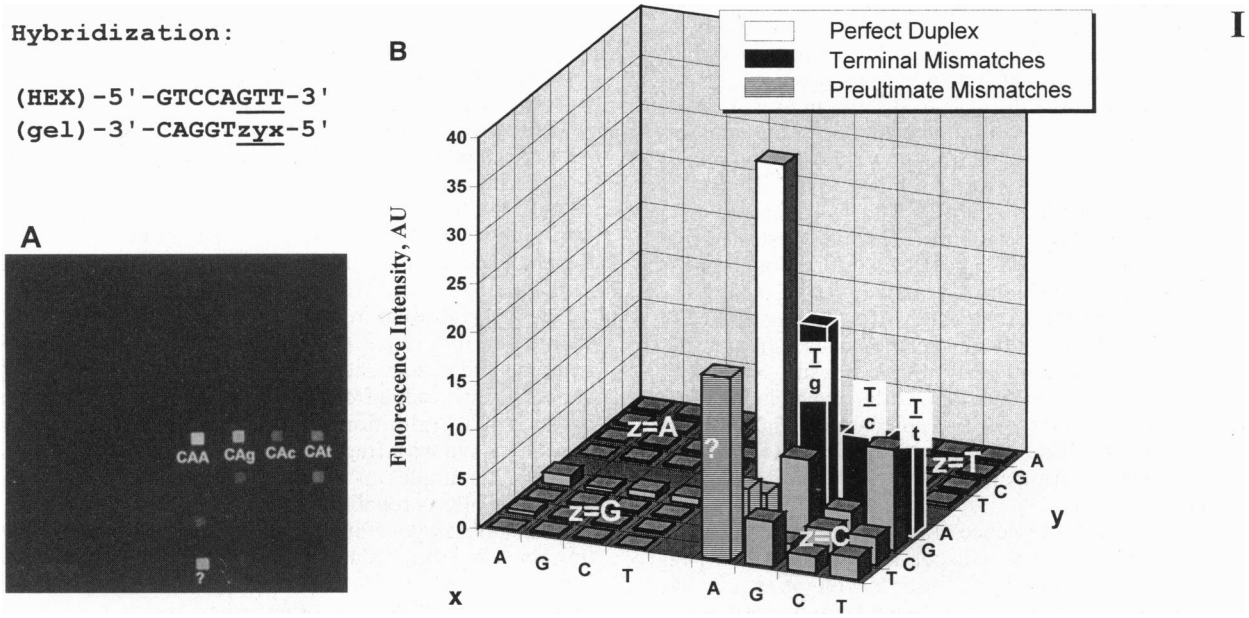

FIG. 2. The effect of mismatches in different positions of the duplex on hybridization efficiency. A 2.2 × 2.2-mm microchip (100 × 100 × 20 μm gel elements, see Fig. 1) contains 64 immobilized octamers with all four bases in the three 5′-terminal positions. The bases in these positions (*x*, *y*, and *z*) are indicated on axes *x*, *y*, and within proper squares *z*. The microchip was hybridized with HEX-5′-GTCCAGTT-3′, a 8-mer fluorescently labeled at the 5′-terminal position. (*A*) Fluorescence microscope image. (*B*) Fluorescence intensities in arbitrary units. The terminal mismatches are indicated above the intensity columns. The ? shows unexpected strong signal from the duplex with T-T mismatch in preultimate position, which has not been observed in other experiments.

dence can be used to equalize the stability of AT- and GC-rich duplexes (14).

A matrix of polyacrylamide gel elements was prepared by gel polymerization on a glass surface and removing the gel from the space between microchip elements by mechanical scribing or by a photolithography and laser evaporation technique (14, 24). A one-pin robot was constructed for automatic transfer of minute solution volumes (Fig. 1). The robot performs 240 transfers per hour of activated oligonucleotide solutions onto the activated micromatrix gel elements. (The 16- and 64-pin robots are under construction to increase the speed of the applications up to 20,000 transfers per hour.) Microchips prepared in this way usually sustain 15, and in some cases 50, rounds of hybridization.

A special two-wavelength epifluorescence microscope coupled with a CCD camera was assembled for image analysis. A special objective with a 3-mm observation field can cover up to 1000 microchip elements within the field. The system provides real-time and simulteneous monitoring of hydridization kinetics at different temperatures for all microchip elements. Parallel two-color monitoring of the hybridization can be carried out with DNA sample labeled with two different fluorophores. Polyacrylamide gels have a low fluorescence background, and the sensitivity of measurements is increased by miniaturization of the microchip. We are able to detect fluorescence down to 2 amol of TMR-labeled target per 40 × 40-μm microchip element.

DNA fluorescently labeled with TMR or HEX and FAM was hybridized with the microchips. Perfect duplexes were easily discriminated from duplexes with internal mismatches as early as in the process of hybridization, before washing, which is convenient for diagnostics. However, for DNA sequencing, more effective discrimination of mismatches (simulteneously for AT- as well as GC-rich duplexes) can be achieved by analyzing the dissociation curves. These curves are plots of the intensity of hybridization signals versus either temperature or time of the washing (14). The optimal range for disrimination lies in the region of the curve where the mismatched signals are close to the background.

Identification of Base Changes in DNA. The reliability of sequence analysis by SHOM depends on how efficiently perfect duplexes can be discriminated from the duplexes with mismatches. We have been studying the effect on duplex stability of different mismatches in various positions. It was shown that the one most difficult to discriminate is a rather stable G-T mismatch in the terminal positions of the duplexes (14, 25–27). Therefore, a microchip with 64 immobilized oligonucleotides with all base variations at three 5′-terminal position was manufactured (Fig. 2). The hybridization of a fluorescently labeled 8-mer with microchip demonstrated that a G-T terminal mismatch decreased the hybridization intensity by about half as compared with a terminal A-T base pair. The difference will be greater if compared with a G-C base pair. All other terminal and internal mismatches cause greater decreases in hybridization intensity. Similar result have been obtained with other mismatches and with mismatches located in other positions of the duplexes (not shown). These results suggest locating the bases to be studied by SHOM inside, rather than in the terminal positions of the immobilized oligonucleotide.

The intensities of fluorescence of the microchip elements in some experiments were normalized. The normalization was carried out by microchip hybridization with an equimolar mixture of all fluorescently labeled 8-mers complementary to the immobilized 8-mers (8).

Diagnostics for β-Thalassemia Mutations. DNA from the blood of β-thalassemia patients has been used in diagnostics for mutations within the first exon and the first intron of the β-globin gene (28). The mutations are IVS-1–1 (G → A), IVS-1–5 (G → C), and IVS-1–6 (T → C) (Fig. 3) (IVS,

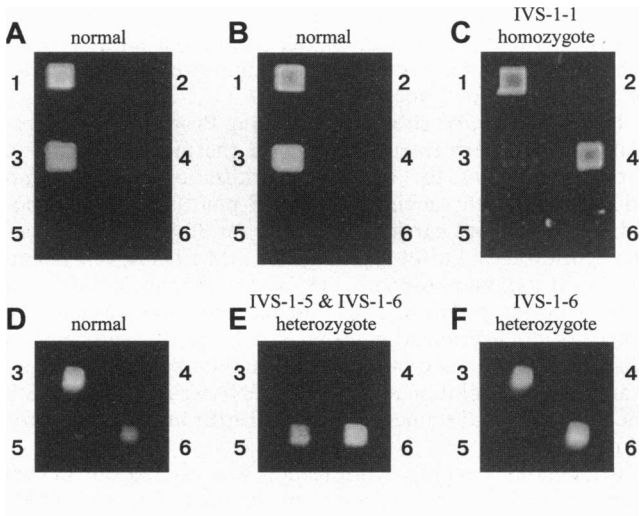

FIG. 3. Diagnostics for β-thalassemia mutations on a microchip. The microchips consisted of six 100 × 100-μm elements (*A–C*) or four 40 × 40-μm elements (*D–F*) of gel-immobilized 10-mers. The 10-mers are indicated on the microchips by the number 1–6 and correspond to different β-thalassemia genotypes. The microchip was hybridized with amplified DNA from patients: (*A*) fragmented 176-bp dsPCR product of normal allele; (*B*) 85-base ssPCR product of the normal allele; (*C*) 85-base ssPCR product of homozygote mutant IVS-1–1 (G → A); (*D*) 32-base ssPCR product of normal allele; (*E*) with 32-base ssPCR product of heterozygote mutant IVS-1–6 (T → C) and IVS-1–5 (G → C); (*F*) with 32-base ssPCR product of heterozygote mutant IVS-1–6 (T → C).

intervening sequence). We used for diagnostics long, fragmented, 176-bp dsDNA and short 85- and 32-base-long ssDNA to decrease the negative effect on the hybridization of hairpin structures and to facilitate DNA diffusion into the gel.

DNA samples were hybridized with 10-mers immobilized on a microchip, having element sizes of 40 × 40 × 20 μm and 100 × 100 × 20 μm. The mutations were located at internal positions of the 10-mers; 10-mers are better hybridyzed than 8-mers, produce more intensive signals, and require less DNA samples.

The hybridization results show (Fig. 3), as expected, a significant difference in hybridization intensities between matched and mismatched duplexes. The size of the microchip elements does not effect the hybridization pattern. Similar patterns of hybridization were obtained with ssDNA 32 and 85 bases long and with fragmented dsDNA 176 bp long. Analysis of over 30 samples of DNA gave similar results. Thus, the procedure allows reliable identification (in a "yes or no" way) of both homozygous and heterozygous β-thalassemia mutations by using short ssDNA and fragmented longer dsDNA. It is apparent that SHOM can be applied for diagnostics of other mutations in the same or other genes.

Applications of Contiguous Stacking Hybridization to Diagnostics. We also applied CSH procedure to microchip diagnostics for β-thalassemia mutations (Fig. 4). Stacking interactions between adjacent bases make a significant contribution to the stability of the DNA duplexes. Five-mers, for example, do not form stable duplexes on DNA. However, a

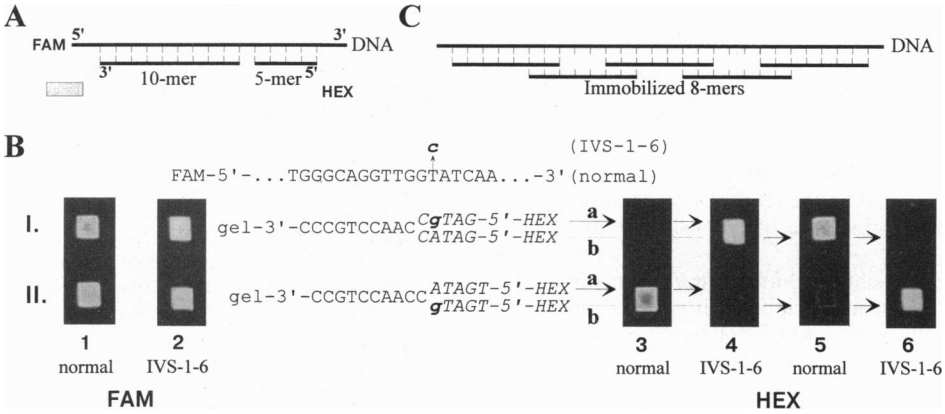

FIG. 4. Diagnostics for mutations by contiguous stacking hybridization. (*A*) Scheme of the contiguous stacking hybridization. (*B*) Diagnostic for β-thalassemia mutations. Two different overlapping decamers close to the mutated base (*I* and *II*) were immobilized on a microchip with elements 100 × 100 μm. The 62-base ssFAM-labeled PCR products, corresponding to a normal allele of β-globin gene (images 1, 3, and 5) and homozygote mutant IVS-1–6 (T → C) (images 2, 4 and 6) were successively hybridized with the microchip. Images 1 and 2 are the hybridizations of normal and mutant FAM-labeled PCR-amplified DNA with the chip, respectively. Then two pools of HEX-labeled pentamers (*a*, TGATA and GATGC; and *b*, GATAC and TGATG) were hybridized consecutively juxtaposed to the immobilized 10-mers *I* and *II*. Two different sets of microscope filters were used for concurrent detection of FAM DNA (images 1–2) and HEX 5-mers (images 3–6). (*C*) Scheme for the use in CSH of the set of 8-mers overlapped by three nucleotides (for explanation, see text).

stable, contiguous, perfect 15-bp duplex is formed by hybridization with DNA of both a 5-mer and an 10-mer adjacently arranged on it (Fig. 4*A*) (5, 14). The 5-mer is stabilized in the contiguous duplex by its stacking with the 10-mer. The experiments with different 5-mers have shown that 5-mers are stabilyzed in duplexes even with weak stacking bases and mismatches in any of the five positions of the 5-mers drastically destabilyze their duplexes.‖

A CSH diagnostic microchip contains two immobilized 10-mers adjacent to the mutated base. The microchip was hybridized with one of two FAM-labeled, 62-base-long, amplified ssDNA of patients corresponding to either normal or homozygote IVS-1–1(G → A) genotypes (Fig. 4*B*). The hybridization was observed with the FAM set of microscope filters (images 1 and 2). Then the DNA 10-mer duplexes were successively hybridized with two pools of HEX-labeled 5-mers. The hybridization was imaged by changing to the HEX set of filters (images 3–6). Each pool contained two overlapping 5-mers juxtaposed to the immobilized 10-mers *I* and *II*. One 5-mer was fully complementary to a normal DNA and another to a mutated DNA. One of the 5-mers in each pool showed intensive hybridization when the mutated 5-mer matched the mutated DNA and the normal 5-mer matched the normal DNA (Fig. 4*B II-3a*, *I-4a*, *I-5b*, and *II-6b*). These 5-mers formed the perfect duplexes with DNA that are stabilized by the stacking interaction with the 10-mer. Another pentamer in each pool mismatched DNA (Fig. 4*B I-3a*, *II-4a*, *II-5b*, and *I-6b*) and was not hybridized at all. Thus, the presence of a single mismatch dramatically weakened the interaction of the 5-mer with the DNA 10-mer duplex. The destabilyzing effect of mismatches grows upon the duplex shortening. Therefore, 5-mers show higher sensitivity than 8-mer, 10-mer, and 13-mer duplexes to the presence of mismatches. It makes the CSH approach more reliable for sequence analysis than the direct SHOM procedure.

The 5-mers can also be ligated to immobilized oligonucleotide enzymatically (29). This can enhance the sensitivity of the procedure by increasing the number of hybridized and chemically fixed 5-mers. However, the high capacity of gel microchips allows us to avoid the ligation. The ligation could complicate experiments, in particular when several rounds of CSH should be performed.

CSH can simplify the large-scale diagnostics of known mutations and gene polymorphism screening. For example, a microchip with 200 8-mers overlapping by 3 nucleotides (Fig. 4C) or 10-mers overlapped by 5 nucleotides covers a 1000-nucleotide-long DNA. This DNA could be hybridized first with a 200-oligonucleotide microchip to find out if there is any mutation in the DNA and, if a mutation is present, to locate the pentanucleotide region with a changed structure. A second round of hybridization with 5-mers corresponding to the known mutations at this 5-mer region will specify the mutation. CSH can be also applied for the identification of unknown base changes with the complete set of all possible ($4^5 = 1024$) 5-mers. Theoretically, the detection of one of the $2^{10} = 1024$ combinations should be carried out only with 10 steps. One of the simple schemes for identification of the changes consists of 20 rounds of hybridization with 20 different pools of 5-mers of the structure with one meaningful base: Annnn, Gnnnn, Tnnnn, Cnnnn, nAnnn, nnAnn, nnnAn, nnnnA, where "n" is a universal base or all four bases that can be hybridized with any base in DNA. The pentamers can be labeled with four fluorophores to mark each meaningful base. The use of a four-color fluorescence microscope to discriminate these four fluorophores can further decrease the number of necessary hybridizations to five steps.

CONCLUSIONS

The results presented here show that oligonucleotide microchips can be effectively used for sequence analysis, diagnostics for genetic diseases, and gene polymorphism studies. Many different oligonucleotides can be immobilized on a tiny surface of a gel microchip in high concentration, which increases the sensitivity of the measurements and the reliability of discrimination of perfect duplexes from mismatched ones, and decreases the needed amount of analysis material.

The robot capacity can be scaled up to manufacture inexpensive microchips containing much larger number of immobilized oligonucleotides—e.g., all 65,536 possible 8-mers. Such oligonucleotides can be used for partial sequencing to check the precision of gel sequencing data and *de novo* sequencing with CSH method. It appears that hybridization, its monitoring, and the whole SHOM procedure can be easily automated. We expect that SHOM supplied with inexpensive microchips

‖Parinov, S., Barski, V., Kirillov, Eu., Timofeev, Ed., Yershov, G. & Mirzabekov, A., CHI Conference on Microfabrication Technology for Research and Diagnostics, Sept. 28–29, 1995, San Francisco.

and hybridization monitoring equipment will find wide application in laboratories and clinics.

The application of oligonucleotide microchips can be further extended to the detection or discovery of microorganisms by hybridization of proper oligonucleotides with ribosomal RNA (30) or DNA of other genes. Immobilized cDNAs or expressed sequence tags have been used for quantitative comparison of the expression of some genes in the *Arabidopsis* genome (31). A similar microchip can increase the sensitivity in these experiments.

Our procedure for microchip manufacturing is versatile and can be applied to produce microchips with various types of immobilized compounds, such as DNA, RNA, proteins, and antibodies, by using more porous gel support. Peptides, chelates specific for different metals, and other chemical substances can be also immobilized on microchips. Such microchips can find a wide application.

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