Visualization of oligonucleotide probes and point mutations in interphase nuclei and DNA fibers using rolling circle DNA amplification

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Rolling circle amplification (RCA) is a surface-anchored DNA replication reaction that can be exploited to visualize single molecular recognition events. Here we report the use of RCA to visualize target DNA sequences as small as 50 nts in peripheral blood lymphocytes or in stretched DNA fibers. Three unique target sequences within the cystic fibrosis transmembrane conductance regulator gene could be detected simultaneously in interphase nuclei, and could be ordered in a linear map in stretched DNA. Allele-discriminating oligonucleotide probes in conjunction with RCA also were used to discriminate wild-type and mutant alleles in the cystic fibrosis transmembrane conductance regulator, p53, BRCA-1, and Gorlin syndrome genes in the nuclei of cultured cells or in DNA fibers. These observations demonstrate that signal amplification by RCA can be coupled to nucleic acid hybridization and multicolor fluorescence imaging to detect single nucleotide changes in DNA within a cytological context or in single DNA molecules. This provides a means for direct physical haplotyping and the analysis of somatic mutations on a cell-by-cell basis.

Fluorescence *in situ* hybridization (FISH) techniques are becoming increasingly powerful analytical tools in both basic science and clinical diagnostics (1, 2). The ability to detect aneuploidy, loss of heterozygosity, chromosomal translocations, or abnormal gene expression levels within cytological specimens can provide important genetic information at the single cell level. However, the current detection sensitivity of standard FISH techniques for DNA targets is generally limited to a few kilobases, although sequences as small as several hundred nucleotides can be visualized on DNA fibers by using tyramide-based signal amplification (3). The detection of smaller target sequences (<100 nts) and the visualization of point mutations or single nucleotide polymorphisms within the cellular context would be highly desirable and open new avenues of investigation using molecular cytogenetics.

Rolling circle DNA amplification (RCA) is a method that can replicate circularized oligonucleotide (ODN) probes with either linear or geometric kinetics under isothermal conditions (4–7). RCA has sufficient sensitivity to detect individual ODN hybridization events (4) and single antigen-antibody complexes (7) on glass surfaces when visualized by fluorescence microscopy. By using ODN probes or antibodies tagged with a DNA primer complementary to a single-strand circular DNA, it was possible to generate long single-stranded DNA molecules containing tandem repeats complementary to the original circle sequence by rolling circle DNA replication. Up to 10^4 copies of the circle could be produced at the site of molecular binding on the glass surface and these RCA products were visualized by hybridization with fluorescently labeled, circle sequence ODNs. In this study, we have extended RCA to the detection of small ODNs hybridized to genomic DNA targets and the visualization of point mutations in cultured cell lines and stretched DNA fibers. Our data further demonstrates the feasibility of haplotyping human cells by *in situ* hybridization of allele-discriminating (AD) ODNs to genomic DNA.

Methods

DNA Sequences and Circular DNA Preparation.
All ODNs were synthesized by the Yale University Critical Technologies facility using phosphoramidite chemistries. The sequences of probe-primer ODNs, P1 "anchor" probes, AD-ODNs, circles, and decorator probes for RCA reactions are listed in Tables 1 and 2.

The probe-primer ODNs and the AD-P2-ODNs were designed to have two 3′ ends. The synthesis of these ODNs was initiated from the 3′ end of the primer sequence with reverse amidites and changed to normal nucleotide amidites after the synthesis of a poly(T) linker centered around a $(CH_2)_{18}$ spacer. Each circle sequence was designed to have a minimum level of secondary structure or cross hybridization to other circles or decorator probes. The decorator probes had sequences identical to that of a segment of their cognate circle, and each was labeled at its 5′ end with a fluorophore (FITC, Cy3, or Cy5). In most experiments two different decorator probes were used to visualize RCA products generated from a single type of circle molecule.

To make single-stranded circles, the linear ODNs were ligated by T4 DNA ligase using guide sequences as templates. The guides contained sequences complementary to the terminal 10 nt from both the 5′ and 3′ ends of the linear ODN. The reaction mixture contained 1 μM linear ODN and 1.2 μM guide ODN in a reaction buffer of 20 mM Tris·HCl, pH 7.5/20 mM KCl/10 mM MgCl$_2$/5 mM DTT/1 mM ATP. The reaction mix was heated to 65°C for 2 min and cooled to room temperature over 30 min. After adding BSA to a final concentration of 0.1 μg/μl and T4 DNA ligase (New England BioLabs) to 7 units/μl, ligation was carried out at 37°C for 2 h. Unligated linear molecules were removed by exonuclease I (Amersham Pharmacia) and T7 gene 6 exonuclease (Amersham Pharmacia) digestion. The circle molecules were further purified by phenol and chloroform extraction, precipitated by the addition of ethanol and centrifugation and then suspended in 10 mM Tris·HCl/0.1 mM EDTA, pH 7.5. The purity of each circle was assessed by electrophoresis on a 15% urea-90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3–PAGE gel.

Cell Lines.
Peripheral blood lymphocytes were obtained from normal laboratory volunteers. Cell lines with mutations at the G542X locus of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (GM11497, heterozygous mutation; GM11496; homozygous mutations), a cell line (CTL2337) with a single C insertion between nucleotides 5382 and 5383 of the *BRCA1* gene, and standard HeLa cell lines, were obtained from the American Tissue Type Collection (Corriel Cell Repositories, Camden, NJ). The BT20 cell line with a homozygous mutation at the A13073C locus of the human *p53* gene was kindly provided by Bonnie King (Yale University, New Haven, CT) while the cell

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Table 1. Physical mapping of three loci in the *CFTR* gene region by RCA

Locus	ODN sequences
Δ508	Probe-primer PRP3 (89): 3′-**CCCTCTTGACCTCGGAAGTCTCCCATTTTAATTCGTGTCACCTTCTTAAA**tttt(CH$_2$)$_{18}$tttttACGTCATCATGAACATTACACGTTCCAC-3′
	Circle3 (78): GTGGAACGTGTAATGTTCATGATGACGTGCATCCTTGACAGCCGATGAGGCTGGCATCCTTGACAGCCGATGAGGCTG
	Decorator probe: Det3-Cy3 (24): 5′-Cy3-GCATCCTTGACAGCCGATGAGGCT-3′
G542X	Probe-primer PRP1 (89): 3′-**GAACCTCTTCCACCTTAGTGTGACTCACCTCCAGTTGCTCGTTCTTAAAG**tttt(CH$_2$)$_{18}$tttttATGATCACAGCTGAGGATAGGACATGCGA-3′
	Circle1 (78): CGCATGTCCTATCCTCAGCTGTGATCATCAGAACTCACCTGTTAGACGCCACCAGCTCCAACTGTGAAGATCGCTTAT
	Decorator probe: Det1c-FITC (18): 5′-FITC-TCAGAACTCACCTGTTAG-3′; Det1d-FITC (18): 5′-FITC-ACTGTGAAGATCGCTTAT-3′
M1101K	Probe-primer PRP4 (89): 3′-**GACGGTTGACCAAGAACATGGACAGTTGTGACGCGACCAAGGTTTACTC**tttt(CH$_2$)$_{18}$tttttCTTGTACATGTCTCAGTAGCTCGTCAGT-3′
	Circle4 (78): ACTGACGAGCTACTGAGACATGTACAAGGAGCAGTCCTGṪCAGCTAGGTCACGGAGCAGTCCTGTCAGCTAGGTCACG
	Decorator probe: Det4-Cy5 (24): 5′-Cy5-GAGCAGTCCTGTCAGCTAGGTCACG-3′

Bold type: probe sequence; lowercase tttt(CH$_2$)$_{18}$ttttt: linker; standard type: RCA primer, circle, and decorator ODN sequences. Note that probe-primer and AD-P2-ODNs have polarity reversal and two 3′ ends.

line FF2914 containing a single mutation at the C3383A locus of the *patched* (Gorlin syndrome) gene was the gift of Allen Bale (Yale University). All cells were cultured in RMP1 media with 10% FCS.

ODN Detection in Interphase Nuclei using RCA. Nonadherent cells (lymphoblast and HeLa cells) were cultured to a cell density of approximately 10^6 cells/ml and the cells collected by centrifugation at 900 rpm for 8 min. Surface adherent cells (GM11496, GM11497, CTL2337, BT20, and FF2914) were grown to semiconfluency, harvested by trypsinization, and pelleted by centrifugation. After the supernatants were removed, the cells were hypotonically swollen in 0.075 M KCl at 37°C for 5–7 min for all cell types except HeLa cells, which required 30–40 min incubation. Fixative solution (methanol/glacial acetic acid, 3:1) was added, and the cells were repelleted and resuspended in fresh fixative. About 10,000–30,000 cells were dropped on a clean slide for *in situ* RCA experiments.

ODN mapping experiments. To detect three target sequences within the CFTR gene, a hybridization mixture containing 30% formamide, 5% dextran sulfate, 2 × SSC, 0.1% Triton X-100, 100 nM of ODN probes PRP1, PRP3 and PRP4, 100 nM of circle 1, circle 3, and circle 4 was added to the slides. After denaturation of the genomic DNA at 95°C for 2 min, hybridization was carried out overnight at 37°C. The slides were washed once 5 min in 10% formamide, 2 × SSC, 0.1% Tween 20 at 42°C, twice 5 min in 2 × SSC, 0.1% Tween 20 at 42°C, and once for 5 min in 50 mM Tris·HCl, pH 7.5/10 mM MgCl$_2$/1 mM DTT. The slides were drained and the RCA solution, containing 50 mM Tris·HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, 100 nM of circle 1, circle 3, and circle 4, 200 ng/μl BSA, 400 μM dATP, dCTP, dGTP, dTTP, and 20 ng/μl Φ29 polymerase, was added to the slides. RCA was performed at 37°C for 60 min. A mixture of 500 nM decorator probes (Det3-Cy3 for ΔF508, Det1c-FITC, Det1d-FITC for G542X, Det4-Cy5 for M1101K) in 2 × SSC, 1% BSA, and 0.1% Tween 20 was applied to the slides. After incubation at 37°C for 30 min, the slides were washed three times for 5 min each in 2 × SSC, 0.1% Tween 20 at 37°C. The DNA

was counterstained in 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and the slides were covered with antifade mounting media (Vectashield, Vector Laboratories) and overlaid with a coverslip. Fluorescent images were captured by using commercial software (Perceptive Scientific Imaging, League City, TX), a Sensysis camera (Photometrics, Tucson, AZ), and an Olympus microscope with multiple excitation and emission filters (Chroma Technology, Brattleboro, VT).

Allele discrimination in interphase nuclei. The cellular DNA was first denatured by incubating slides in 70% formamide, 2 × SSC, pH 7.0 at 70°C for 90 s. Hybridization and DNA ligation were carried out simultaneously by adding to each slide 40 μl of a reaction mixture containing 20 mM Tris·HCl (pH 8.3), 10 mM MgCl$_2$, 25 mM KCl, 0.5 mM NAD, 0.01% Triton X-100, 10 nM P1 ODN, 10 nM wild-type (wt) P2 ODN, 10 nM Mu P2 ODN, 100 ng/μl BSA, and 0.5 units/μl Ampligase. The solution was overlaid with a 22 × 30 mm coverslip and sealed with rubber cement, and the slide was incubated for 2 h at either 60°C or 65°C, depending on the ligation specificity of the different probes. Ligation temperatures for maximal allele discrimination were established by solution studies using synthetic ODN targets. Ligation reactions with P1 and P2 probes were run at temperatures ranging from 55°C to 65°C, and the specificity of each probe set was determined by analyzing the ligation products on a 15% urea-90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3-PAGE gel. Optimal temperatures for allele discrimination of the *CFTR*, *BRCA-1*, *p53*, and *patched* gene mutations studied were 60°C, 60°C, 65°C, and 65°C, respectively. After a 2-h incubation at the designated temperature, the slides were washed once (5 min) in 25% formamide, 2 × SSC, 0.1% Tween 20 at 42°C, and twice (5 min) in 2 × SSC Tween 20 at 42°C. Wt and mutant (mu) circles (10 nM each) were added to the slides in a buffer containing 40 mM Tris·HCl (pH 7.5), 10 mM MgCl$_2$, 50 mM NaCl, and 0.1% Tween 20 and incubated for 30 min at 42°C. After removing excess circles by washing in RCA buffer (40 mM Tris·HCl, pH 7.5/10 mM MgCl$_2$/50 mM NaCl/5 mM DTT/100 ng/μl BSA), 40 μl of RCA buffer containing in addition 400 μM

Table 2. Sequences for discrimination of mutations by RCA

Gene	Anchor probes (P1) and AD (P2) probes
CFTR	P1 5′-AACGAGCAACTGGAGGTGAGTCACACTAAGGTGGAAGAG-3′; P2Wt 3′-**GTTCTTGATATAACAGAAAG**tttt(CH$_2$)$_{18}$tttttATGATCACAGCTGAGG-ATAGGACATGCGA-3′; P2Mu 3′-**TTTCTTGATATAACAGAAAG**tttt(CH$_2$)$_{18}$tttttACGTCGTCCGTGCTAGAAGGAAACACGCA-3′
P53	P1 5′-GTTGAGGGCAGGGGAGTACTGTAGGAAGAGGAAAGGAGACA-3′; P2Wt 3′-**TTCTACAAAACGGTTGACC**tttt(CH$_2$)$_{18}$tttttATGATCACAGCTGAGG-ATAGGACATGCGA-3′; P2Mu 3′-**GTCTACAAAACGGTTGAC**tttt(CH$_2$)$_{18}$tttttACGTCGTCCGTGCTAGAAGGAAACACGCA-3′
Patched	P1 5′-ACACGGCGCCATCCAGGACGGGTGCAAACATGTGCTCCAG-3′; P2Wt 3′-**GGTGAGACGACCCTC**tttt(CH$_2$)$_{18}$tttttATGATCACAGCTGAGGATA-GGACATGCGA-3′; P2Mu 3′-**TGTGAGACGACCCTC**tttt(CH$_2$)$_{18}$tttttACGTCGTCCGTGCTAGAAGGAAACACGCA-3′
BRCA1	P1 5′-ATTCTCTTGCTCGCTTTGGACCTTGGTTTCTTCCATT-3′; P2Wt 3′-**GGGTCCTGTCTTTCTAGAAG**tttt(CH$_2$)$_{18}$tttttATGATCACAGCTGAGGAT-AGGACATGCGA-3′; P2Mu 3′-**GGGGTCCTGTCTTTCTAGAA**tttt(CH$_2$)$_{18}$tttttACGTCGTCCGTGCTAGAAGGAAACACGCA-3′
Circle sequences:	Wt: CGCATGTCCTATCCTCAGCTGTGATCATCAGAACTCACCTGTTAGACGCCACCAGCTCCAACTGTGAAGATCGCTTAT
	Mu: GCGTGTTTCCTTCTAGCACGGACGACGTATATGATGGTACCGCAGCCAGCATCACCAGACTGAGTATCTCCTATCACT
Decorator probe sequences:	Wt: Det1a-Cy5: 5′-Cy5-TCAGAACTCACCTGTTAGACGCCA; Det1b-Cy5: 5′-Cy5-GCTCCAACTGTGAAGATCGCTTAT
	Mu: Det2a-Cy3: 5′-Cy3-TATATGATGGTACCGCAGCCAGCA; Det2b-Cy3: 5′-CCAGACTGAGTATCTCCTATCACT

Bold type: probe sequence; lowercase tttt(CH$_2$)$_{18}$ttttt: linker; standard type: RCA primer, circle, and decorator ODN sequences. Note that probe-primer and AD-P2-ODNs have polarity reversal and two 3′ ends.

dATP, dCTP, dGTP, 200 μM biotin-dUTP, 200 μM dTTP, 4 μM single-stranded DNA-binding protein, and 1 μM Sequenase was added to the slides and incubated for 30 min at 37°C. The slides were washed three times for 5 min in 2 × SSC, 0.1% Tween 20 at 42°C, and the RCA products were detected by incubation with a mixture of decorator ODNs (Det): 0.5 μM each Det la-Cy5 and Det 1b-Cy5 for wt alleles and 0.5 μM each Det 2a-Cy3 and Det 2b-Cy3 for mu alleles in 2 × SSC, 1% BSA, 0.1% Tween 20 for 30 min at 37°C. After washing three times for 5 min in 2 × SSC, 0.1% Tween 20 at 37°C, the RCA products were collapsed to a point source by incubating with 5 μg/ml Avidin in 2 × SSC, 1% BSA, 0.1% Tween 20 for 30 min at 37°C. Slides were washed three times at 37°C, 5 min each, in 2 × SSC, 0.1% Tween 20, and DNA was counterstained with 2 μg/ml DAPI. Fluorescent images were captured as described.

RCA Reactions on Genomic DNA Fibers. Human genomic DNA fibers were stretched from freshly cultured normal peripheral blood lymphocytes and GM11496 cells (homozygous G542X mutation). About 30,000 cells were dropped and evenly distributed on a clean slide. The slide was vertically dipped into a lysis buffer (100 mM Tris·HCl, pH 7.5/100 mM EDTA/0.5% SDS) and incubated for 10 min. An equal amount of 100% ethanol was added on top of the lysis buffer. After 10-min incubation, the slide was taken out of the solution at a constant slow speed and kept in a vertical position for about 1 min. Finally, the slide was dried through 70%, 90%, and 100% ethanol.

ODN mapping experiments. Two CFTR locus plasmid P1-derived artificial chromosome (PAC) clones, PAC20f22 and PAC17c02, were hybridized first to DNA fibers as reference markers. DNAs prepared from PAC20f22 and PAC17c02 were labeled with biotin and digoxigenin, respectively, by nick-translation. Approximately 200 ng of each probe in 20 μl hybridization mixture (50% formamide, 10% dextran sulfate, 2 × SSC, 100 ng/μl salmon sperm DNA, and 100 ng/μl human Cot-1 DNA) was denatured by boiling for 5 min. A DNA-fiber preparation was denatured by incubation in 70% formamide, 2 × SSC at 70°C for 2 min, and dehydrated through ice-cold ethanol series (70%, 90%, and 100%) 3 min each and air-dried. The hybridization mixture was applied to the DNA-fiber preparation and incubated overnight at 37°C. The slide was washed three times for 5 min in 2 × SSC at 37°C, and then the 3′ ends of the two PAC probe fragments were blocked by incubation with 50 μl of a ddNTP blocking mixture containing 50 mM Tris·HCl (pH 7.5), 40 mM KOAc, 10 mM MgCl₂, 10 mM DTT, 100 μg/ml BSA, 40 μM ddNTP, and 0.1 unit/μl Klenow at 37°C for 30 min. The slides were washed three times for 5 min in 2 × SSC at 37°C, and the three RCA probes then were hybridized to the DNA fibers. A hybridization mixture, containing 30% formamide, 5% dextran sulfate, 2 × SSC, 0.1% Triton X-100, 100 nM of PRP1, PRP3, and PRP4, 100 nM of circle 1, circle 3, and circle 4, was added onto the slides and incubated overnight at 37°C. The slides were washed once 5 min in 10% formamide, 2 × SSC, 0.1% Tween 20 at 42°C, and twice 5 min in 2 × SSC, 0.1% Tween 20 at 42°C. After preincubation of the slides in 40 mM Tris·HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 5 mM DTT for 5 min, a RCA reaction mixture, containing 40 mM Tris·HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 5 mM DTT, 100 nM of circle 1, circle 3, and circle 4, 200 ng/μl BSA, 400 μM dATP, dCTP, dGTP, 200 μM dTTP, 200 μM Biotin-dUTP, 4 μM single-stranded DNA-binding protein, and 1 μM T7 Sequenase, was added to the slides. RCA was performed at 37°C for 30 min. A mixture of 500 nM decorator probes (Det3-Cy3 for ΔF508, Det1c-FITC, Det1d-FITC for G542X, Det4-Cy5 for M1101K) in 2 × SSC, 1% BSA, and 0.1% Tween 20 was applied to the slides. After incubation at 37°C for 30 min, the slides were washed three times for 5 min each in 2 × SSC, 0.1% Tween 20 at 37°C. Finally, the biotinylated and digoxigenin-labeled PAC probes were detected with FITC and rhodamine/Texas red, respectively, using three successive layers of antibodies
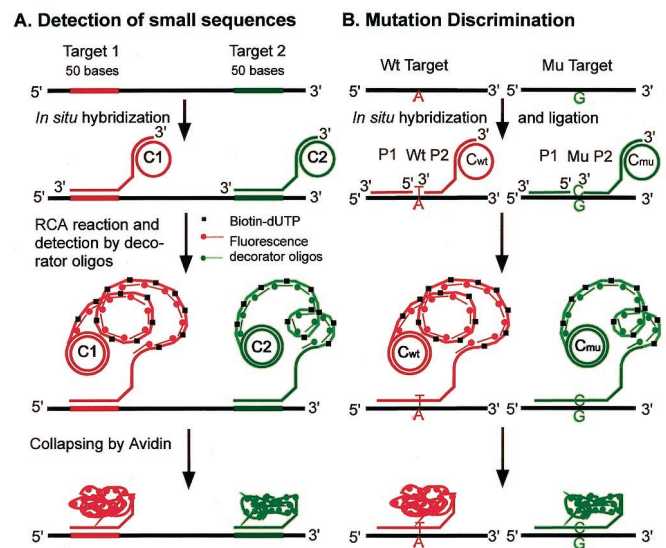

Fig. 1. Schematic for detecting small target sequences (*A*) or point mutations (*B*) using RCA. See text for description.

as follows: (*i*) 2 μg/ml avidin-FITC + 5 μg/ml sheep anti-digoxigenin rhodamine; (*ii*) 2 μg/ml biotinylated goat-anti-avidin + 5 μg/ml rabbit-anti-sheep Texas red; and (*iii*) 2 μg/ml avidin-FITC + 5 μg/ml goat-anti-rabbit Texas red. All antibodies were in a solution of 2 × SSC, 1% BSA, 0.1% Tween 20, and incubated at 37°C for 30 min. All washes were three times in 2 × SSC, 0.1% Tween 20 at 37°C. DNA fibers was counterstained in 2 μg/ml DAPI for 5 min, and the slides were covered with antifade Vectashield (Vector Laboratories). The RCA signals were captured by a charge-coupled device camera using filters for DAPI, FITC, Cy3, Cy5, and Texas red and combined in PHOTOSHOP software (see Fig. 2c). To evaluate the specificity and efficiency of the RCA signals on DNA fibers, 50 images of PAC fiber-FISH were taken.

Allele discrimination assays. Experiments designed for allele discrimination at the G542X locus did not use the PAC prehybridization step outlined above. Instead, the P1 anchor ODN and the pair of P2 AD-ODNs for the G542X locus were cohybridized with the 50-mer non-AD-ODN probes for the Δ508 and M1101K loci, which acted as reference markers. The hybridization, ligation, RCA reactions, washes, and signal detection conditions were carried out as described for the detection of G542X mutations in interphase cells.

Results

Probe Design. We have used two different probe designs for the visualization of small target sequences and the discrimination of point mutations in genomic DNA of cytological specimens using RCA. These are illustrated schematically in Fig. 1 *A* and *B*, respectively. When allele discrimination is not required, a single ODN probe can be used. This probe has one portion, generally 40–50 nt long, complementary to the target sequence and a second portion, 24–28 nt long, that functions as a primer to initiate rolling circle replication of the circular DNA template required for the RCA reaction. To stabilize the association of the RCA products on target DNA molecules and to increase detection efficiency, this probe-primer ODN is synthesized with two 3′ ends (see *Methods*). The polarity reversal permits the 3′ end of the target complementary sequence to be extended at the same time as the primer sequence, potentially resulting in longer, more stable probe-target hybrids via primer extension *in situ* synthesis (8).

In contrast, allele discrimination assays use three ODNs for each locus analyzed. An anchor probe (P1), 30–40 nt long, is synthesized
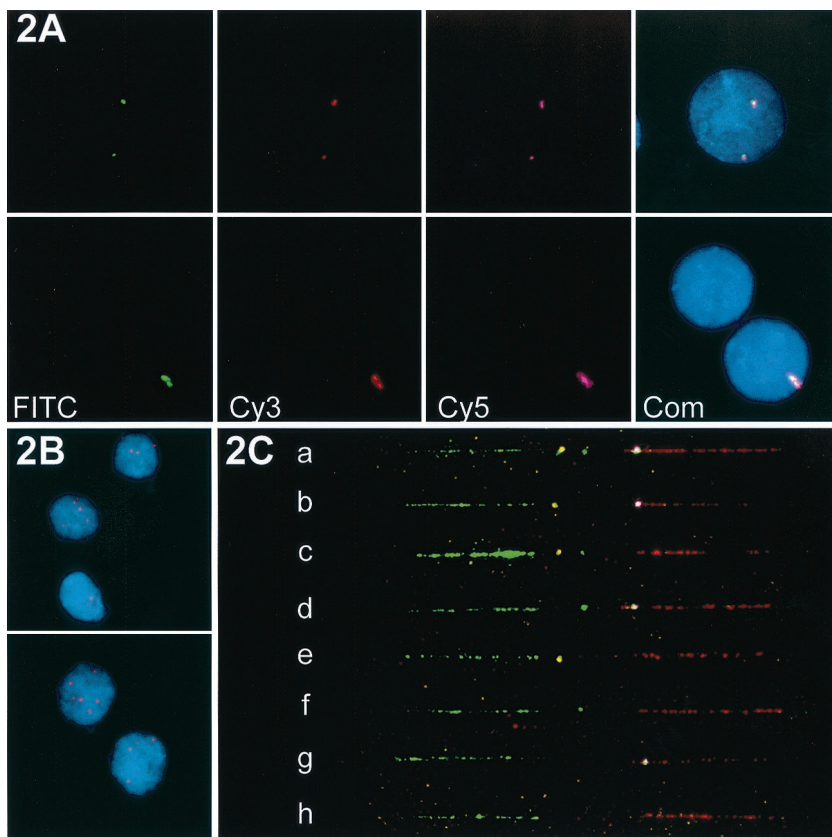
Fig. 2. Visualization of ODN probes hybridized to 50-mer target sequences in the CFTR gene in interphase nuclei and DNA fibers. (*A*) RCA detection of probes targeted to the G542X locus (FITC), the Δ508 locus (Cy-3), and the M1101K locus (Cy5) in normal human lymphocytes. Merged images (com) show that signals from all three loci colocalize. (*B*) Hybridization of the Δ508 locus probe to nuclei of HeLa cells. (*C*) Cohybridization of two PAC clones (extended green and red signals) with Δ508 (yellow), G542X (green), and M1101K (white) oligomer probes. Eight patterns (*a–h*) of hybridization are produced (see text).

such that its 5′ end is immediately adjacent to the nucleotide in the target to be interrogated as to its wt or mu status. The two AD-ODNs (P2 oligos) have inverted (3′-3′) polarity with a target complementary sequence, 15–20 nt long, that differs only at the 3′ terminal nucleotide. The primer portions of the two P2 ODNs are attached to the target portions via a spacer and the primers contain sequences complementary to two different circular DNAs, both 78 nt long. After hybridization of a mixture of P1 and P2 probes to target DNA, the samples are subjected to a DNA ligation reaction. Because ligation of the P1 oligo to a perfect match P2 is much preferred to a mismatch ligation event, perfect match P1-P2 ligations predominate. Hybrids made with P2 ODNs are less stable than those of P1-P2 ligation products and are disrupted under the wash condition used.

In both assay formats subsequent incubation of a circular DNA (or a mixture of circles complementary to primer sequences specific for the wt or mu allele) with a strand-displacing DNA polymerase and a dNTP mixture results in the production of RCA products that can be visualized with fluorophore-labeled decorator probes complementary to the appropriate (locus- or allele-specific) RCA products.

Visualization of 50-Nt Sequences Within the *CFTR* Gene. To test the ability of RCA to detect small genomic DNA sequences within interphase nuclei and stretched DNA fibers we chose three 50-bp targets at the Δ508, G542X, and M101K mutation loci within the *CFTR* gene. The sequences of these ODNs, with associated RCA primers, RCA circles and the fluorescently labeled decorator probes are given in Tables 1 and 2. All components first were validated in solution RCA reactions driven by either ϕ29 or Sequenase DNA polymerases at 37°C, and the RCA products were analyzed on a 0.9% alkaline agarose gel. No RCA products were produced in the absence of a circle, a probe-primer ODN, a polymerase, or when incorrect primer:circle pairs were used

(data not shown). The DNA generated by sequence matched RCA reactions was variable in length (generally 15–50 kb) yet some molecules as long as 400 kb were observed (data not shown) when the RCA products were stretched on silane coated slides (9), and the DNA fibers were stained with YoYo-1 (Molecular Probes). Although Sequenase produces more RCA products per unit of incubation time, RCA synthesis by ϕ29 polymerase is more robust and reproducible, largely because of variations in the quality of different commercial preparations of single-stranded DNA binding protein, which Sequenase requires for activity but ϕ29 does not. Nevertheless, both polymerases generated *in situ* data like that illustrated in Figs. 2–4.

Strong RCA signals were observed when the three 50-mer ODN probes were hybridized to either interphase nuclei or stretched DNA fibers from normal diploid lymphoblast cell lines (Fig. 2*A* and *C*, respectively). In Fig. 2*A*, the RCA products from the Δ508, G542X, and M11101K probes were detected by Cy3-, FITC-, and Cy5-labeled decorator ODNs, respectively. Because the known physical distances between the three probes is only 50 kb, below the spatial resolution limits of interphase FISH, the signals from each ODN probe are colocalized in the merged image as expected. In a typical experiment, approximately 20% of the nuclei gave the expected two signals from each of the three oligos, 36% gave all three signals from only one chromosomal site whereas 44% of the nuclei showed no RCA signals at all. In addition, approximately 25% of the chromosomal CFTR loci gave signals from only one or two of the ODN probes. For every 100 G_1 cells imaged one would expect 600 signals (two gene copies $\times$ three probes/gene) if hybridization and probe detection were 100% efficient. The overall RCA detection efficiency at the Δ508, G542X, and M1101K loci averaged 37%, 45%, and 35%, respectively.

The 50-mer probe for the Δ508 locus also was tested alone on a preparation of HeLa cell interphase nuclei, which are known to have varying levels of polyploidy (10). The results, shown in

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Fig. 2*B*, show that a variable number of RCA signals are observed per cell with over 90% of the nuclei exhibiting at least one signal. A total of 277 RCA signals were counted in 70 cells, an average of 3.96 per cell.

Human genomic DNA fibers from cultured normal lymphocytes were prepared as described in *Methods*. To more rapidly find the *CFTR* gene locus on the fiber preparations and to facilitate quantitative analysis two PAC clones from the *CFTR* gene were cohybridized with the three ODN probes. These clones flank the three ODN loci and were labeled with FITC (PAC22f20, green) and Texas red (PAC17c02, red), respectively. Signal amplification by RCA was done only for the ODN probes. Image analysis after cohybridization of the five probes revealed eight different hybridization patterns (Fig. 2*C*). All eight patterns (100% of 50 fibers scored) had signals from the two PAC clones, whereas 78% of the fibers had RCA signals from one or more of the three ODN probes; 8% had signals with all three ODNs (pattern *a*); 34% had signals from two of the three (patterns *b*, *c*, and *d*) and 36% had one of the ODN signals (patterns *e*, *f*, and *g*). The overall efficiency in detecting RCA signals at the Δ508, G542X, and M1101K loci were 48%, 44%, and 36% respectively. It is interesting to note that the relative physical distance in microns between the ODN signals on the fibers correspond to the known physical map separation in kbps (15 kb and 35 kb).

Allele Discrimination in Interphase Nuclei and DNA Fibers Using RCA.
We next tested the ability of RCA to visualize point mutations in interphase cells and DNA fibers by using the P1/P2 probe-ligation scheme illustrated in Fig. 1*B* and described above. The sequences of the P1 and the two AD-P2 ODNs designed to detect mutations at the G542X locus of the *CFTR* gene are given in Table 2. Cell lines that were known to be wt, homozygous mu, or heterozygous at this loci were cultured, hyponically swollen, and fixed by methanol-acetic acid treatment. Hybridization with a mixture of P1 and P2 AD-ODNs and DNA ligation reactions were carried out simultaneously at 60°C for 2 h (see *Methods*). After removing excess probe, slides were incubated with the pair of AD circles, and then subjected to RCA reactions followed by detection with fluor-labeled decorator ODNs.

Fig. 3*A* shows the results obtained with nuclei from the three different cell lines. The wt cell line, normal peripheral blood lymphocytes, shows two green nuclear signals; the homozygous mu cell line, GM11496, shows two red nuclear signals, and the heterozygous cell line, GM11496, gave one green and one red nuclear signal. In each cell line, 100% of the nuclei exhibiting two signals gave the expected genotype. This experiment has been repeated more than 10 times with similar results. The efficiency of RCA signal detection, however, has varied considerably from experiment to experiment. In the worst-case scenario, 5% or less of the cells gave any RCA signal whereas at the other extreme up to 80% of cells were RCA-positive at one or both chromosomal loci. On average, RCA signals were generated from both *CFTR* genes in ≈21% of the cells and from a single copy of the gene in 30–40% of the cells. Some of the potential reasons for this variability are considered in *Discussion*.

None of the cell lines used in any of our experiments were synchronized, therefore each cell population is expected to have cells in the G$_1$, S, or G$_2$ phase of the cell cycle. G$_2$ cells can be identified readily as four RCA signals can be generated per locus in each nucleus, usually as a pair of closely juxtaposed (gemini) signals. Examples of G$_2$ nuclei from wt, homozygous mu, or heterozygous cells are shown in Fig. 3*A Lower*.

Detection of the G542X mutation also was done on stretched DNA fibers prepared from normal lymphocytes and the GM11496 cell line homozygous for the G542X mutation (Fig. 3*B*). Here, P1 and AD P2 ODNs were cohybridized with the nonallele discriminating Δ508 and M1101K 50mer probes previously used for small target detection (Fig. 2 *A* and *B*). After

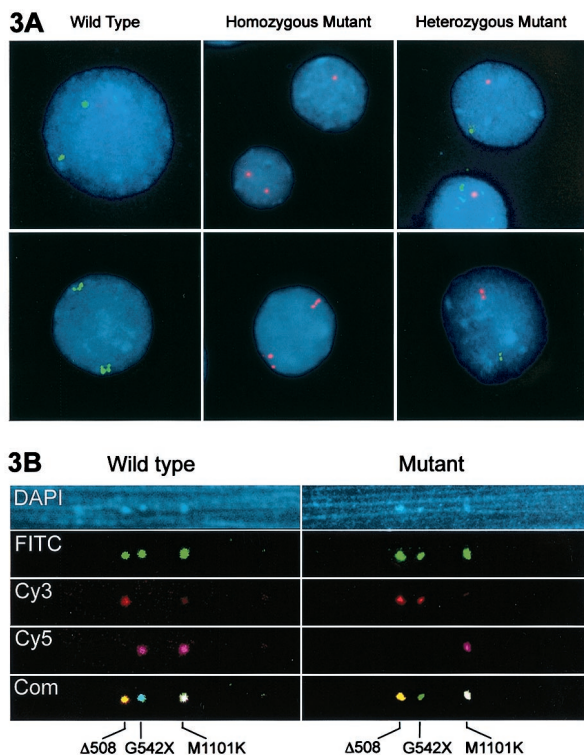**Fig. 3.** RCA detection of wt and mu alleles at the G542X locus of the CFTR gene. (*A*) Nuclei from wt, homozygous mu, and heterozygous cells in G$_1$ phase (*Upper*) or G$_2$ phase (*Lower*) of cell cycle. (*B*) Discrimination of wt and mu alleles of the G542X locus on stretched DNA fibers.

hybridization, ligation, and RCA signal amplification, the RCA products were visualized by hybridization with fluor-labeled decorator ODNs. RCA products at each of the three CFTR loci
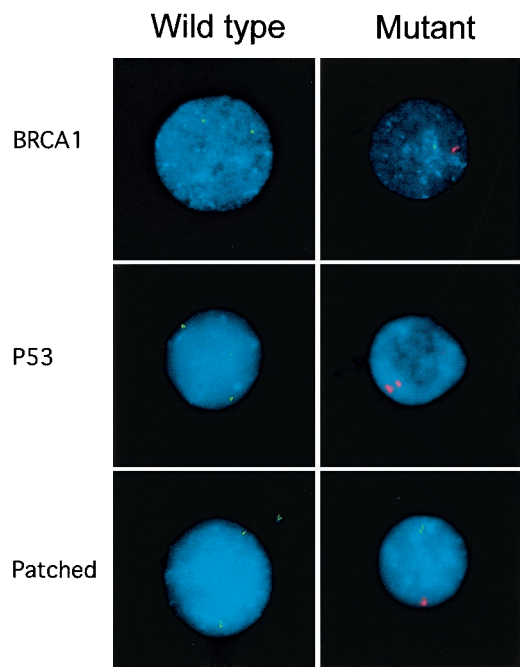

Fig. 4. RCA detection of wt and mu alleles at the A13073C locus of the *p53* gene, the 5382C ins locus of the *BRCA* gene, and the C3383A locus of the *patched* (Gorlin syndrome) gene.

can be seen on both the DAPI and the FITC images (decorator probes for each locus were FITC-labeled). The wt $\Delta 508$ and M1101K loci were detected by using Cy3- and Cy5-labeled probes, respectively. The wt G542X allele is labeled with Cy5 while the mu allele is labeled with Cy3. The merged image (Com) shows a yellow-blue-white hybridization pattern when the G542X allele is wt and a yellow-green-white pattern when G542X is a mu allele. Because flanking PAC clones were not used in these experiments, quantitation of the overall detection efficiency at each locus was not assessed rigorously. It was apparent, however, that wt and mu alleles at the G542X could be readily detected as many fibers showing the hybridization profiles illustrated in Fig. 3B were observed.

To determine whether mutations could be detected in other genes, P1/P2 probe sets were prepared for the A13073C locus in *p53*, the 5382Cins locus in *BRCA-1*, and the C3383A locus in the *patched* gene (Table 2). Tissue culture cells containing either wt or mu alleles in each of these genes were analyzed by using AD-RCA reactions. As shown in Fig. 4, wt and mu alleles could be discriminated in each cell line. As with the CFTR gene, 100% of the nuclei exhibiting RCA signal from both genes correctly identified the expected genotype. Again, the efficiency of detecting both gene copies in the nucleus of individual cells varied from experiment to experiment. Despite this caveat, the data clearly indicate that genetic mutations can be visualized in a cellular context on a cell-by-cell basis.

Discussion

We have demonstrated that both unimolecular and bipartite DNA probes possess the specificity required to detected short gene sequences, as well as single base changes in the context of the entire human genome, as evidenced by the absence of nonspecific RCA signals in cell nuclei. The observation of four distinct signals in the nuclei of cells in G_2 (Fig. 3B) attests to the power of this method. At the present time the frequency of RCA signals in interphase nuclei fluctuates between 20% and 55% of potentially accessible DNA target sequences. Many interesting applications, such as the detection of somatic mutations, would require an efficiency of 80% or better. Genotyping of single cells from blastomeres, for selection of healthy embryos, would require an efficiency of 100%.

Part of the variability in detection efficiency is undoubtedly due to differential chromatin condensation and the accessibility of targeted loci to either the ODN probes or required RCA reagents (circles, polymerase or ligase, decorator ODNs). In support of this interpretation, we note that detection efficiency on DNA fibers and nuclear halo preparations (data not shown) is generally higher than that on interphase nuclei. Furthermore, none of the probes described here have produced an RCA signal when hybridized to metaphase chromosomes, which have the highest level of DNA condensation. Various cellular pretreatment protocols, and/or additional probe designs will need to be evaluated to increase the overall efficiency of small probe detection and *in situ* mutational analysis.

The ability to map precisely the location of short sequences and point mutations in DNA fibers opens avenues for studying HLA haplotypes, haplotypes based on single nucleotide polymorphisms (SNPs) or even Ig gene rearrangements by direct visualization of single DNA strands. Although SNP genotyping via ODN arrays or mass spectrometry is now a mature technology, SNP haplotyping is still based on statistical analysis of linkage probability. Detection and ordering of SNPs on individual DNA molecules via RCA should provide a means of establishing haplotypes by direct physical measurements. The capability for visualization of single base changes at the single cell level or in DNA fibers will also be particularly useful in studies involving gene therapy, site-directed mutagenesis with triplex ODNs (11, 12), or gene correction using DNA-RNA chimeras (13, 14). Other potential applications of *in situ* RCA on fiber preparations include the study of methylation status, using DNA treated with bisulfite, to distinguish cytosine from 5-methyl-cytosine converted to uracil. An alternative approach to detection of methylation is the use of immuno RCA (7) using RCA primers coupled to antibodies specific for 5-methyl cytosine.

A highly sensitive reporter system for *in situ* RNA detection reported recently (15, 16), based on tyramide amplification, has been used as a one-color or two-color detection system. The RCA system reported here offers a higher potential for multiplexing because it is entirely DNA-based and does not require different enzymes. Using as many as 10 different circular ODNs, and specific complementary primers for each circle, the RCA system can be modified to allow the detection of multiple targets simultaneously by using spectrally resolvable fluorescent dyes or quantum dots (17, 18).

A major challenge for the future is the extension of DNA genotyping by *in situ* RCA to histological sections. Our attempts to use the methods for DNA detection in formalin-fixed tissue have so far resulted in a significantly lower frequency of signals. Other interesting challenges for the future included the detection of rare RNA targets in cells or tissues or the detection of low-copy number viral genomes. In many of these applications, surface-anchored RCA can deliver the ultimate sensitivity in FISH assays, i.e., the power to discriminate a single base change in a target sequence, in the context of the entire human genome.

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