Mutation detection and single-molecule counting using isothermal rolling-circle amplification

Paul M. Lizardi[1], Xiaohua Huang[2], Zhengrong Zhu[2], Patricia Bray-Ward[2], David C. Thomas[3] & David C. Ward[2]

Rolling-circle amplification (RCA) driven by DNA polymerase can replicate circularized oligonucleotide probes with either linear or geometric kinetics under isothermal conditions. In the presence of two primers, one hybridizing to the + strand, and the other, to the – strand of DNA, a complex pattern of DNA strand displacement ensues that generates 10^9 or more copies of each circle in 90 minutes, enabling detection of point mutations in human genomic DNA. Using a single primer, RCA generates hundreds of tandemly linked copies of a covalently closed circle in a few minutes. If matrix-associated, the DNA product remains bound at the site of synthesis, where it may be tagged, condensed and imaged as a point light source. Linear oligonucleotide probes bound covalently on a glass surface can generate RCA signals, the colour of which indicates the allele status of the target, depending on the outcome of specific, target-directed ligation events. As RCA permits millions of individual probe molecules to be counted and sorted using colour codes, it is particularly amenable for the analysis of rare somatic mutations. RCA also shows promise for the detection of padlock probes bound to single-copy genes in cytological preparations.

Nucleic-acid amplification technology has greatly increased our ability to ask detailed questions about genotype or transcriptional phenotype in small biological samples, and has provided the impetus for many significant advances in biology, especially in the field of genetics. Recently, the utility of circularizable oligonucleotides, called 'padlock probes', was demonstrated in the detection of repeated alphoid sequences in metaphase chromosomes[1]. The high sequence specificity of padlock probes, and their potential for the discrimination of point mutations *in situ*[2] prompted us to explore the amplification of circular DNA. The ability of small circular oligonucleotides to serve as templates for DNA polymerases has been documented[3,4]. We have devised alternative methods for ligation-dependent circularization of padlock probes, as well as a method employing preformed circular probes, all of which can be exploited for allele discrimination. These methods have been coupled to isothermal nucleic-acid amplification reactions based on a rolling-circle replication mechanism.

We used a novel geometric hyperbranched RCA (HRCA) reaction to detect point mutations in small amounts of human genomic DNA in solution. Furthermore, linear RCA, using preformed circles as universal amplification templates, was used as a sensitive reporter system to visualize single hybridization/ligation events on surfaces that contain site-addressable probes. The linear RCA reporter system provides a new paradigm for genetic analysis at the molecular level, using a variety of formats ranging from surface-immobilized DNA to cytological specimens.

Linear amplification of allele-specific circularized probes

The circularizable probes described[1] contain two adjacent probe sequences of 20 bases. To increase specificity, we chose to use circularizable probes that hybridize to the target leaving a small gap of 6–10 nt. This gap can be filled by a short, allele-specific oligonucleotide with a 5′ phosphate (Fig. 1*a*) that fits exactly within the gap, completing a stacked duplex structure that is ligated with DNA ligase to form a closed padlock probe (Fig. 1*b*). Alternatively, the gap may be filled by DNA polymerase (see below).

We designed an allele-discriminating probe for a 46-nt target sequence in the *CFTR G542X* gene locus (Fig. 2*a*). The circularizable probe and either of the two alternative allele-specific gap oligonucleotides, each 8 nt, were incubated with an artificial DNA target to investigate sequence discrimination in a ligation reaction. The formation of the circularized oligonucleotide was assessed by its characteristic electrophoretic mobility (Fig. 2*b*). Using wild-type target, the slow-migrating circularized oligonucleotide is observed only when the corresponding wild-type gap oligonucleotide is present. The converse results were obtained using mutant target DNA. The ligation reaction was also strictly dependent on correct sequence complementarity of the gap probe sequence for another three probes of similar design (at two other loci in *CFTR*, and at one locus in *OTC*, data not shown).

An 18-base oligonucleotide complementary to the circularizable probe (Fig. 1*b*) was added after probe ligation to serve as a primer for RCA (Fig. 1*c*). To catalyze this linear RCA reaction, we used the DNA polymerase of phage ø29 (ref. 5), a highly processive enzyme that displays strand-displacing activity in the absence of additional proteins or cofactors[6]. We followed the time course of primer extension by gel electrophoresis in a denaturing alkaline agarose gel. After 10 min, the DNA product was larger than the 23-kb DNA marker (Fig. 3). The observed reaction rate is approximately 53 nt per second, a value consistent with previously published data[6] on the replication of single-stranded M13 DNA by ø29 DNA polymerase. Sequence analysis of cloned RCA products revealed that the amplified DNA contained the sequence expected for each different target-dependent, allele-specific, circle-ligation event (see Methods).

[1]*Department of Pathology, and* [2]*Department of Genetics, Yale University School of Medicine, 333 Cedar St., New Haven, Connecticut 06520, USA.* [3]*Oncor, Inc., 209 Perry Parkway, Gaithersburg, Maryland 20877, USA. Correspondence should be addressed to P.M.L. (e-mail: paul.lizardi@yale.edu) or D.C.W. (e-mail: ward@biomed.med.yale.edu).*

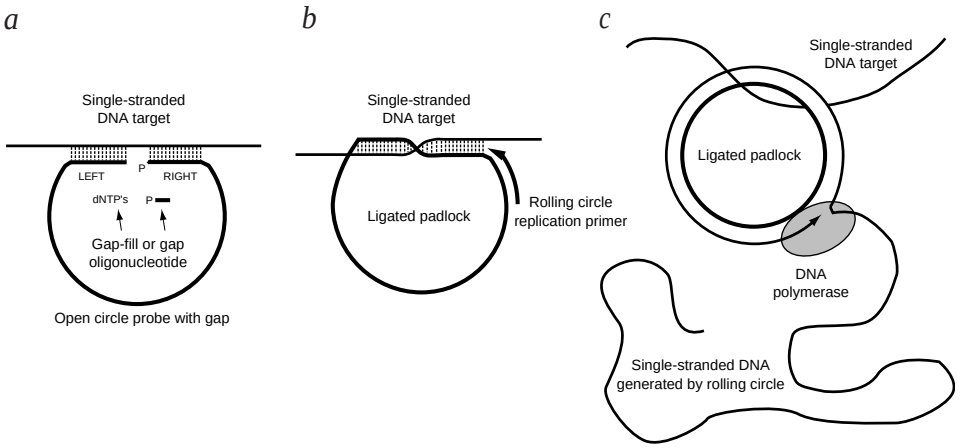

Fig. 1 Probe circularization by ligation, and amplification by a rolling circle-reaction. ***a***, Circularizable probe with a small gap, which is to be filled by binding and ligation of a small phosphorylated oligonucleotide, or by DNA polymerase incorporation of dNTP's proceeding from the 3′-OH end of the probe, and terminating at the junction with the 5′ end of the probe, with concomitant ligation by DNA ligase. ***b***, Ligated (padlock) probe, and binding of complementary primer for rolling circle amplification. The primer 3′ end is located five or six bases away from the last paired base in the hybridized probe arm. ***c***, Rolling-circle amplification of a padlock probe, catalyzed by a strand displacing DNA polymerase.

Allele discrimination using HRCA and genomic DNA

An alternative probe design for a 46-nt target sequence in the *CFTR G542X* gene locus (Fig. 2*c*) was used in this experiment. It consisted of an 18-base probe sequence at the 3′ end of the circularizable probe, and a 21-base probe sequence at the phosphorylated 5′ end. A gap of seven bases is formed, which will be filled by extension of the 3′-hydroxyl end of the probe using *Thermus flavus* DNA polymerase[7]. The gap-fill reaction, followed by ligation, generates a circular probe harbouring a faithful copy of a small segment of the target sequence. Using artificial DNA targets, the formation of circles after incubation in the presence of DNA polymerase and DNA ligase was found to be quantitative as assessed by gel electrophoresis (Fig. 2*d*). As expected, when polymerase was added in the absence of ligase, the probe was extended, but not circularized.

To facilitate allele discrimination in genomic DNA, we designed a second oligonucleotide primer to bind specifically to the tandem DNA generated by RCA of the gap-fill probe. This primer, which is not complementary to the first primer, will bind to each complementary sequence in the tandem single-stranded DNA, initiating sequential primer extension reactions (Fig. 4). As each extending primer runs into the product of a downstream primer, strand displacement will ensue, generating single-stranded tandem repeats of the sequence of the original circularized probe. This displaced strand will in turn contain multiple binding sites for the first RCA primer. Thus, alternate-strand copying and strand displacement processes generate a continuously expanding pattern of DNA branches connected to the original circle. Strand displacement also generates a discrete set of free DNA fragments comprising double-stranded pieces of the unit length of a circle, and multiples thereof. We call this expanding cascade of strand displacement and fragment-generation events 'DNA hyperbranching', and the special rolling circle amplification driven by two primers 'Hyperbranched-RCA' (HRCA).

HRCA reactions were performed using the exonuclease(–) variant of Vent DNA polymerase, in the presence of phage T4 gene 32 protein[8] at 62–66.5 °C. When seeded with known amounts of preformed circular oligonucleotides, the reactions generate a large DNA output within 60–90 min. The size distribution of the amplified DNA comprises a ladder of bands starting at unit circle length, and extending in discrete increments to several thousand nucleotides (Fig. 5*b*), as predicted by the DNA hyperbranching

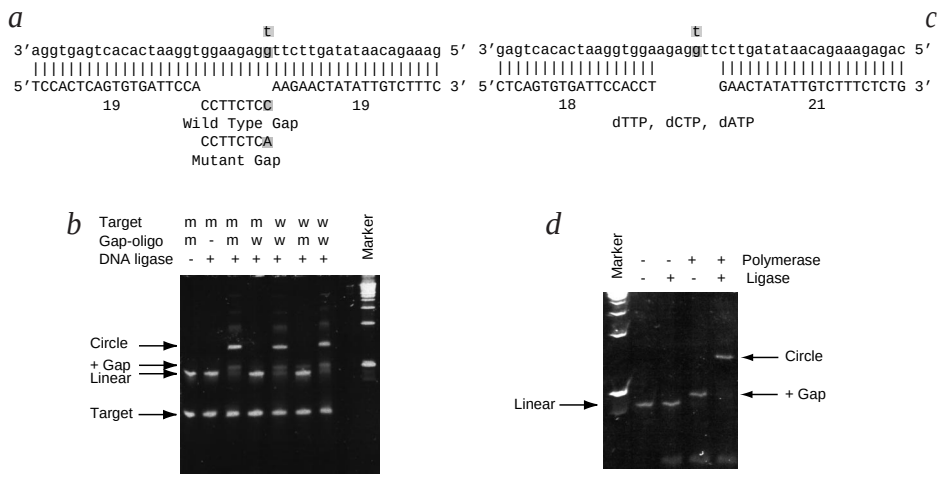

Fig. 2 Ligation of circularizable probes by Ampligase, a thermostable DNA ligase. ***a***, Sequence of the hybridizing arms of a circularizable probe, and two alternative 8-base gap probes designed for the *CFTR G542X* locus. The probe consists of a 19-base probe sequence at the 3′ end, and a 19-base probe sequence at the phosphorylated 5′ end. The probe is 85 nt (see sequence in Methods), and 47 nt of the sequence, constituting the non-hybridized backbone, are arbitrary. An 8-base oligonucleotide that is phosphorylated at the 5′ end is designed to fit in the gap by base pairing with the target. The base at the 3′ end of the 8-nt gap probe is either C or A, corresponding to the complement of the wild type or mutant allele, respectively. The artificial target was 53 nt, but only 46 bases are shown in the illustration. Published studies on *T. thermophilus* DNA ligase[12] have demonstrated that discrimination is highest when the mismatched base is at the 3′ end of the oligonucleotide to be ligated, as is the case for the probes shown here. ***b***, Analysis by gel electrophoresis of circularizable probes ligated with artificial DNA targets in the presence of different gap oligonucleotides. The gels were standard 8% polyacrylamide in Tris-Borate EDTA containing 8M urea, and staining was performed with SYBR-Green II (Molecular Probes). Letters indicate mutant (m) or wild type (w) sequences. The marker lane contains a commercial DNA ladder, where the fastest-migrating band is 100 bases. The ligation efficiency of the non-cognate gap oligonucleotide is low even for the 5′ terminus, which in all cases contains correct sequence complementarity (notice the very low amount of product of size 85+8=93 nt, indicated by the +Gap arrow). This may be explained by the ligation temperature, which is higher than the T_m of the gap oligonucleotide and may hinder the formation of a stable double helix for mismatched DNA. ***c***, Sequence of the hybridizing arms of a circularizable gap-fill probe designed for the *CFTR G542X* mutation locus. The artificial target was 53 nt, but only 46 bases are shown in the illustration. The complete sequence of the 89-nt circularizable probe is in the Methods section. Gap-filling occurs in the presence of dTTP, dCTP and dATP. ***d***, Analysis by gel electrophoresis of an 89-base circularizable probe that was gap-filled and ligated with artificial DNA targets in the presence or absence of DNA polymerase and DNA ligase, as indicated by plus (+) and minus (–) signs. The first lane contains a 100-bp DNA marker. Electrophoresis was performed as in (*b*).

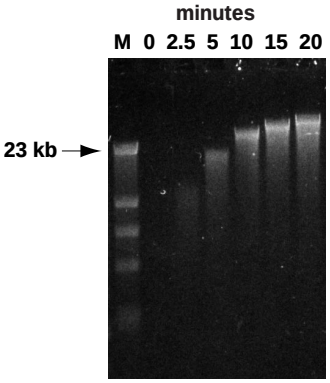

Fig. 3 Analysis of RCA products. Time course of amplification of circularized probes, catalyzed by ø29 DNA polymerase. Circularized padlock probes were prepared as in Fig. 2*b*. Aliquots of the RCA reaction were taken at the times indicated, denatured in alkaline buffer and analysed on a 0.7% alkaline agarose gel. The lane marked as 'M' contained phage lambda DNA digested with restriction endonuclease *Hin*dIII. The largest DNA fragment of 23 kb is indicated by the arrow.

diagram (Fig. 4). The data demonstrates that the HRCA reaction catalyzed by exo(–) Vent DNA polymerase can be initiated with as few as 20 molecules of closed circles, and after 90 min, produces sufficient material for detection. We estimate that each individual circle in the reactions initiated with 20 molecules generated at least 5×10^9 copies of the 96-base repeat during HRCA.

In order to demonstrate the use of HRCA in gene detection, we analysed genomic DNA from tissue-culture cells of defined genotype in an allele-specific amplification assay. Human lymphocytes that were either wild-type, homozygous or heterozygous for the *G542X* locus were used as a DNA source. An assay was performed where *G542X* probes of the gap-fill design (Fig. 2*c*) were extended and ligated using genomic DNA targets, and amplified using HRCA. The amplification reactions employed one common primer, complementary to the backbone sequence of the probe, and either of two allele-specific reverse primers, designed to be selective for each of the two alternative alleles (Fig. 5*a*). The discrimination of single-base changes using allele-specific primers is well documented for PCR (ref. 9). We used primers that contain a deliberately mismatched base at position –3 relative to the 3′-hydroxyl end, in addition to the 3′-terminal discriminating base, in order to improve specificity. After amplification, the DNA was cleaved with a restriction endonuclease whose recognition sequence occurs once in the probe, to generate a single band of repeat-sized fragments. Amplified DNA was generated only in those reactions in which the correct allele-specific primer was used (Fig. 5*c*). In heterozygous DNA samples, the intensity of the band of amplified DNA was somewhat lower, as expected for the halved gene dosage. In a sample containing a mixture of 90% wild-type DNA and 10% mutant DNA, a weaker band corresponding to the mutant allele was observed with good reproducibility in different experiments (lane 11, Fig. 5*c*). We used HRCA for the amplification of another 10 different DNA targets with excellent results, using either Vent DNA polymerase or Bst large fragment DNA polymerase (D.C.T., unpublished data).

Detection of individual ligation events by single-molecule counting

RCA can be used as a reporter system for quantifying hybridization/ligation events on a glass surface by single-molecule analysis. We prepared slides containing an oligonucleotide probe (P1) specific for a 39-base sequence adjacent to the G542X locus of the CFTR gene. The probe contained a free 5′ phosphate, and was bound covalently to the glass surface *via* a reactive 3′-amino group. Two additional probes (P2wt, P2mu) were designed, capable of being ligated to either the wild-type or mutant locus, with precise base stacking continuity with the 5′ end of the P1 probe. An allele-specific base was located at a 3′-hydroxyl terminus in these probes, for optimal discrimination in the ligation step; the opposite end of these probes comprises a coded primer sequence (corresponding to one of two alternative primers) with a free 3′-OH terminus, obtained by reversal of backbone polarity during chemical synthesis. The ligation of P1 with the allele-specific probes P2wt or P2mu (Fig. 6) generates a surface-bound oligonucleotide with a free 3′ terminus, competent for coded priming of an RCA reaction. We used two different pre-formed circular DNA templates for RCA signal coding, one designed to be complementary to the primer sequence of P2wt, the other to the P2mu primer. One of these two circular molecules will enable RCA of its cognate primer, in the event that the complementary primer becomes covalently bound to the surface by a ligation reaction. In the absence of ligation, no priming can occur. The DNA generated by RCA is labelled with

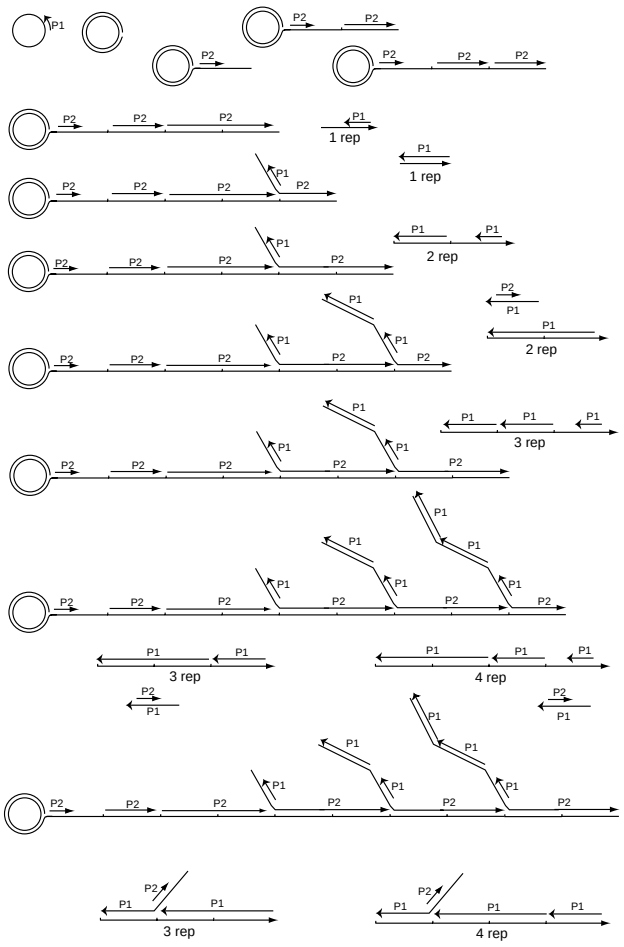

Fig. 4 Rolling-circle amplification of a circularized probe using two primers. The first primer (P1) initiates an RCA reaction, and the reverse primer (P2) binds to each tandem repeat generated by the rolling circle. Multiple priming events are initiated by P2 as the original RCA strand elongates. As these priming events elongate and generate displaced DNA strands, new priming sites for the first primer (P1) are generated. To follow the sequence of strand displacement events, note that as the reverse primer P2 binds to the fifth repeat, the primer at the third repeat begins to displace a branch; subsequently, as P2 binds to the seventh repeat, the elongating strand at the fifth repeat begins to displace a branch, and so forth. By the time a reverse primer binds to the tenth repeat, the DNA product already contains three growing branches. New primer extension events initiated in released DNA molecules also generate branches, as shown at the bottom of the figure. As the displaced DNA becomes completely double-stranded, it accumulates in fragments of unit length containing one, two, three or four repeats (shown as: 1 rep, 2 rep, 3 rep, 4 rep). Thus, in the presence of a circular template, the two primers generate a self-propagating, ever-increasing pattern of alternating strand-displacement, branching and DNA fragment release events, which we call hyperbranching.

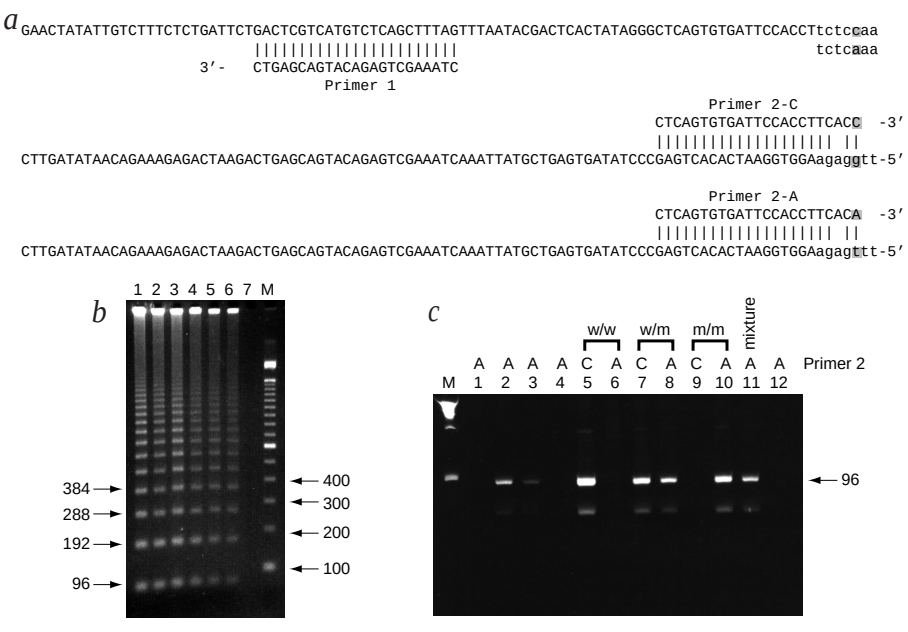

Fig. 5 HRCA of circularized DNA and its use in allele detection. ***a***, Design of primers used to amplify an 89-base probe that had been extended by DNA polymerase copying of 7 nt (shown in small type) from the *G542X* target region, and circularized by DNA ligase. Primer 1 is the same for both reactions, and primer 2-C and primer 2-A are allele-specific reverse primers, binding to unique sequences in the complementary strands generated by copying of the circular probe. The calculated T_m of primer 1 is 68 °C, whereas the calculated T_m for the 20 bases that hybridize upstream of the –3 mismatch in primers 2-C and 2-A is 66.8 °C, using 50 mM salt and 0.1 µM oligonucleotide as parameters for the nearest neighbour T_m calculations[19]. ***b***, Reaction products generated at 65.5 °C with different inputs of artificially made circular DNA, and analysed in a non-denaturing 2% agarose gel. Lanes 1–7 were seeded, respectively, with 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10^1, 2×10^1 (repeat), or zero molecules of *G542X* circularized mutant probe. Lane 8 contains a ladder of markers in multiples of 100 bp. ***c***, Detection of the *G542X* point mutation. DNA samples from homozygous wild type, heterozygous or homozygous mutant cultured lymphocytes were denatured by heating for 4 min at 96 °C, and mixed with the gap-fill open circle probe for the *G542X* locus shown in 6A. After filling with *T. flavus* DNA polymerase and ligation with Ampligase, a 4-µl aliquot of the ligated material was incubated in an HRCA reaction containing the appropriate set of two primers. The net DNA input for each amplification reaction consisted of 28 ng of genomic DNA, which corresponds to 17,000 copies of the gene locus. Amplified DNA was cleaved with *Alu*I and analysed by electrophoresis in a non-denaturing 8% acrylamide gel. Letters indicate mutant (m) or wild type (w) DNA haploid complements. The lane labelled M contains a 100-bp marker DNA. Other lanes contain HRCA reactions initiated with different inputs. Lanes: 1, zero DNA input; 2, 2×10^4 closed circles; 3, 2×10^3 closed circles; 4, ligation with phage lambda DNA; 5, 6, ligation with GM07828(+/+) DNA; 7, 8, ligation with GM11497B(+/–) DNA; 9, 10, ligation with GM11496(–/–) DNA; lane 11, ligation with an artificial 10:1 mixture of GM07828(+/+) DNA and GM11496(–/–) DNA; lane 12, ligation in the absence of genomic DNA. The faint band below the main 96-base Alu fragment corresponds with the primer-induced deletion fragment repeats, which comprise a full unit repeat minus the 22-base segment that separates the 5′ ends of the first primer and the reverse primer (Fig. 6*a*). Four different batches of T4 Gene 32 protein have been used successfully for HRCA reactions. Although the batch used in (c) was less stimulatory than the other three batches, it still allowed detection of the low-abundance mutant allele in lane 11.

fluorescent DNP-oligonucleotide tags that hybridize at multiple sites in the tandem DNA sequence. The 'decorated' DNA, labelled by specific encoding tags, is then condensed into a small object by cross-linking with a multivalent anti-DNP IgM. The wild-type specific primer generates RCA products which can hybridize to fluorescein-labelled DNP-oligonucleotide tags, whereas the mutant RCA products hybridize to Cy3-labelled DNP-oligonucleotides. The acronym for the process of condensation of amplified circles after hybridization of encoding tags is CACHET.

We performed an assay to measure the ratio of mutant to wild type strands at the G542X locus in genomic DNA samples that had been constructed to simulate the presence of rare somatic mutations. DNA mixed in different ratios was amplified by PCR, and hybridized on slides with immobilized P1 probes, in the presence of an equimolar mixture of P2wt and P2mu probes in solution. After ligation of the P2 probes, signals were generated by RCA-CACHET and imaged as described in Methods. The images show many hundreds of fluorescent dots (Fig. 7*a*–*d*) with a dia-

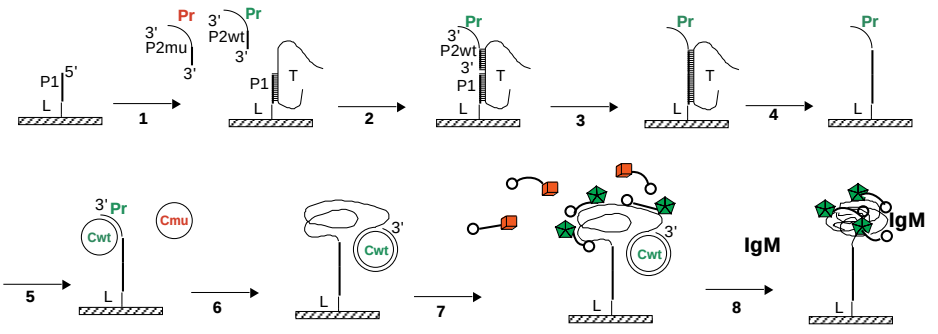

Fig. 6 Design of the RCA-CACHET ligation-dependent assay using immobilized DNA probes. A derivatized glass surface contains an oligonucleotide probe (P1) which is immobilized *via* a spacer (L), bound covalently to the glass. P1 is designed to form 39 bp with the G542X target, and the 5′ terminus of P1 contains a 5′-phosphate to permit ligation. This orientation is preferred because it eliminates the possibility of nonspecific priming by the 3′ end of P1, which could otherwise interact with the circular oligonucleotide templates used for RCA. A set of two allele-specific oligonucleotide probes (P2mu and P2wt) that are linked to different primer sequences (Pr, green or red) is allowed to hybridize with a DNA target (T). These probes, present in solution, are designed to hybridize to a 20-base sequence of the target adjacent to P1, with their 3′ end precisely in stacking contact with the 5′ end of P1, so that P1 and P2 may be ligated. P2-wt contains a 3′-terminal 'G', whereas P2mu contains a 3′-terminal 'T'. Both P2wt-Pr and P2mu-Pr contain at the opposite end a sequence that does not hybridize with the target, so that it may serve as a primer. Therefore, these molecules are synthesized with reversed backbones, and have two 3′ ends. After hybridization of the cognate probe to target, which in the case shown is a wild-type sequence, a thermostable DNA ligase catalyzes the joining of P2wt-Pr to the immobilized P1 probe. Subsequently, the targets, excess probes and any other molecules that are not covalently linked to the solid support are removed by stringent washing. A mixture of two types of circular oligonucleotides, Cwt and Cmu, are hybridized to the primer (Pr, green), which in the case illustrated is complementary to Cwt. The primer is then extended by RCA, using a circular CTwt oligonucleotide as a template. The elongated DNA molecule is then decorated by hybridization of DNP-oligonucleotide tags that harbour either fluorescein or Cy3 fluorescent labels. In the case shown, only the green tags are competent for binding, as the amplified circle only contains sequences complementary to the green tags. The amplified DNA product is finally condensed with anti-DNP IgM, forming a small globular DNA:IgM aggregate that contains green fluorescent tags.

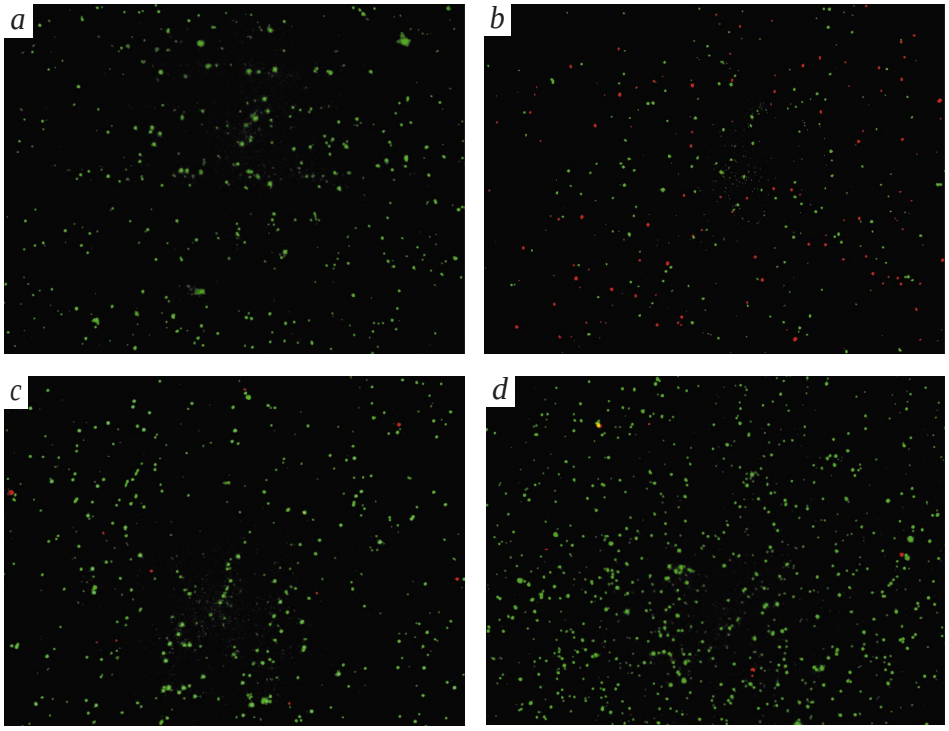

Fig. 7 Detection of individual ligated probe molecules on glass slides by RCA-CACHET. ***a*–*d***, Fluorescent imaging of individual signals generated by surface-immobilized probes. Experiments with different PCR amplicons are shown in each panel, as follows: (*a*) mu:wt=0:1; (*b*) mu:wt=1:1; (*c*) mu:wt= 1:25; (*d*) mu:wt=1:100. Only a small area from each dot on the array is presented. The fluorescent signals from RCA-CACHET products tagged with fluorescein-labelled and Cy3-labelled detector oligos were acquired separately in grey scale using two filter sets (excitation/emission: 455±35 nm/530±15 nm bandpass filters for FITC and 546±5 nm/570±5 nm bandpass interference filters for Cy3). Each image shown is the superimposition of two separate images, with FITC and Cy3 signals pseudocoloured in green and red, respectively. The orange dot in Fig. 6*d* is generated by superimposition of a green signal and a red signal, and probably arises from two RCA extensions occurring in close proximity. ***e***, Table of observed counts of individual fluorescent signals pooled from images of several microscope fields.

e

	Ratio mu/wt	Red counts	Green counts	Total counts	Cy3/FITC ratio
a	0:1	9	4613	4622	1:513
b	1:1	2093	2315	4408	1:1.1
c	1:25	107	2758	2865	1:26
d	1:100	46	4799	4845	1:104

meter of 0.2–0.5 μ, which are generated by single molecules of condensed DNA. The ratio of Cy3-labelled to fluorescein-labelled dots (Fig. 7*e*) corresponds closely with the known ratio of mutant to wild type strands, down to a value of 1/100. A few red signals were generated by pure wild-type DNA (ratio = 1/513), and we interpret these as resulting from mismatch ligation events, which are expected to occur with a frequency of 1/500 to 1/1500 when using wild-type *Thermus Thermophilus* DNA ligase[10].

Detection of padlock probes in cytological samples

As linear RCA was shown to permit analysis of single DNA primers immobilized on glass, we tested this amplification reaction for the detection of padlock probes *in situ*. In spite of the efficiency of surface RCA, padlock probes represent a serious challenge for amplification. Differential chromatin condensation and probe accessibility within a cellular milieu could present obstacles for a rolling-circle reaction. Indeed, attempts to detect the *CFTR* locus in metaphase chromosomes by RCA of ligated padlock probes were unsuccessful. To test the feasibility of padlock probe detection on deproteinized DNA, we ligated the G542X padlock probe (Fig. 2*a*) on a sample of wild-type human genomic DNA which had been immobilized and denatured on a polylysine coated glass slide, at a density of approximately 480 haploid genomes per square mm. An RCA reaction was carried out for 22 min *in situ* to amplify the ligated padlock probes, incorporating BUDR as a hapten into the ssDNA product. The ssDNA was detected by binding of biotinylated anti-BUDR IgG, followed by fluorescent labelling with FITC-avidin. Although most of the ssDNA molecules generated by RCA were condensed into small compact objects, we observed many extended molecules, which were several microns long, lying on the surface of the polylysine-coated glass slide, which contains residual positive charges (Fig. 8*a*). The same padlock probe, with a gap oligonucleotide specific for the wild-type *G542X* locus, was ligated on salt-extracted nuclei prepared by the 'halo' method[11,12]. By carrying out RCA for only 15 min to generate shorter DNA strands, and using the same hapten incorporation and labelling protocol, discernible strands of RCA product originating from padlock probes ligated on the perinuclear DNA halo were observed with very low frequency (Fig. 8*b*). On standard slides, which are not charged, many small, discrete signals were observed inside nuclei or on the perinuclear DNA halo. More than 99.9% of these signals consisted of DNA that was tightly condensed into a small fluorescent dot,

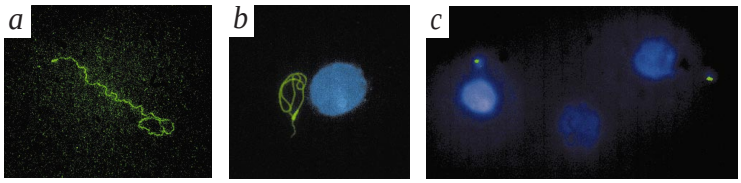

Fig. 8 Detection of padlock probes amplified by RCA on cytological preparations. ***a***, Observation of non-condensed rolling circle DNA product from a padlock probe specific for the *CFTR G542X* wt locus, labelled with BUDR as a hapten, lying on the surface of a polylysine-coated slide. ***b***,***c***, Observation of partly condensed (*b*) and fully condensed (*c*) rolling-circle amplification signals generated by *CFTR G542X* wt padlock probes that had been hybridized to nuclear 'halo' cytological preparations (see text for details). Images were photographed with a 63× objective using a Zeiss epifluorescence microscope with a cooled CCD camera. The frequency of nuclei displaying one or two RCA signals in halo preparations of wild-type cells was 197/2182 for the *G542X* locus wt probes, 1936/2573 for *deltaF508* locus wt probes. When we probed the X-linked *OTC* with a wild-type probe, we observed only one or zero signals per male cell nucleus, and no male nuclei with two signals (data not shown).

due to the cross-linking effect of avidin:biotin interactions (Fig. 8*c*). Not all nuclei displayed signals, and most nuclei contained a single signal; only a few nuclei had two signals. The frequency of nuclei displaying one or two RCA signals in several halo preparations that contained at least 2000 cells per slide is provided (legend, Fig. 8). Although this initial data is highly encouraging, the variable detection efficiency of probes specific for different loci suggests that our current protocols for *in situ* RCA do not provide optimal reagent access to all regions in chromatin.

Discussion

We have demonstrated three alternative strategies for allele discrimination utilizing ligation of circularizable DNA probes and rolling-circle replication. All three strategies employ RCA or HRCA for signal generation, and the assays on surfaces additionally employ the novel CACHET principle for signal condensation and identification. The polymerase mediated 'gap-fill' reaction is preferred over the 'gap-probe' ligation reaction for solution studies of complex genomes. This is because the gap oligonucleotides, which cannot be washed away in a solution assay, must be used at relatively high concentrations for the ligation step and often interfere with the HRCA reaction, inducing the formation of amplicon artifacts. The gap-ligation reaction is considered preferable for allele analysis of DNA in cytological specimens. Here, the specificity of ligation should be enhanced relative to probes without a gap because three different sequence recognition events and two independent ligation events must occur before 'padlock' closure. The third assay method, the ligase-mediated extension of an oligonucleotide linked to a solid surface, provides a novel approach to quantify hybridization/ligation events for single molecules and to score rare somatic mutations.

There are other potential uses for the gap-fill reaction, in which target-complementary sequences are incorporated into circles by copying and covalent closure. In principle, any DNA sequence thus captured into a circular DNA may be amplified by RCA or HRCA. We envision applications of this reaction in situations where it is desirable to interrogate the sequence incorporated into a padlock probe at some point after RCA. Additional experiments will be required to determine if longer sequences, such as microsatellite repeats, can be copied into circularizable probes for amplification and analysis. As there is little likelihood that rolling-circle amplification will modify the number of repeats incorporated into a circularized probe[3], direct measurement of repeat size would be feasible.

For reasons that we do not yet understand, ø29 DNA polymerase is a poor catalyst for HRCA. HRCA is catalyzed by exo(–) Vent DNA polymerase, by the large fragment of Bst DNA polymerase[13] and by the Sequenase 2.0 variant of T7 DNA polymerase. It had been shown by others[3,4] that Sequenase supports rolling-circle amplification of circular oligonucleotides, albeit relatively slowly. The efficiency of RCA and HRCA reactions is increased by the addition of proteins that bind ssDNA. The addition of *E. coli* single-strand binding protein (SSB) stimulates Sequenase-catalyzed HRCA, whereas phage T4 gene-32 protein stimulates Vent exo(–) catalyzed HRCA. In contrast, Bst large fragment DNA polymerase catalyzes HRCA efficiently in the absence of protein cofactors. Although Sequenase is a suitable catalyst for linear RCA, its rapid polymerization rate in the presence of SSB is not necessarily optimal for HRCA. Many potential priming sites may not be used, as they are rapidly occupied by competing extension reactions. Paradoxically, the theoretical HRCA reaction yield should be somewhat higher at relatively slower polymerization rates (in the range of 16.5 nt per second, as reported for Vent DNA polymerase[14]), because this rate permits a more efficient utilization of priming sites. Our current data demonstrate that the HRCA reaction is most efficient when catalyzed by thermostable strand-displacing enzymes. Additional work will be required to reveal which DNA polymerase is the ideal catalyst for HRCA, although the reaction yield already compares very favourably with PCR.

It is worth noting other unusual features of HRCA. When allele-specific primers are used, there are some mis-priming events, but the strands generated by such events will incorporate the incorrect base only in the primer itself, and not in the several hundred tandem copies that result from each primer extension. Thus, in contrast with PCR, non-specific priming is a non-propagating event in HRCA. For applications that do not require allele-specific primers, two invariant primers may be designed, so that circles with different DNA inserts are replicated with a universal primer set, facilitating reproducible amplification kinetics.

The amplification of small circularized oligonucleotides by linear RCA is rapid, technically simple and the amplified DNA will not diffuse away from the site of synthesis. To date, we have used over 20 different circularized oligonucleotides in the range of 60–110 bases, and have not observed any variability in amplification due to sequence content. RCA products may be detected by incorporating haptens or fluors directly, and it is possible to use circles with biased base compositions to obtain differential labelling. A larger range of labelling combinations is attainable by RCA-CACHET. The two-circle/two-primer signal generating system (Fig. 6) is extensible to any number of probes, and retains the use of the same pair of circular amplicons. Two different allele-discriminating primers are required for each mutational locus being assayed. Although photolithographic DNA microarray technology enables massively parallel assays for mutation detection, altered bases are only detectable if they constitute a sizable fraction of the DNA population. Thus, such arrays are well suited for the detection of germline mutations, but not rare somatic mutations. Ligation-mediated RCA extends the utility of assays based on surface-bound oligonucleotides by permitting the detection of infrequent mutations in the presence of a large excess of wild-type DNA. The detection limit for such mutations will be determined by the stringency of the DNA ligation reaction, which could be improved by using new ligase variants[10]. The single-molecule counting approach promises to be both sensitive and linear in its response to target concentration. Individual immunoglobulins tagged with rolling-circle primers can be detected by RCA-CACHET. This approach is being used by our laboratory to detect single molecules of antibody bound to antigen (Z.Z., unpublished observations). A logical extension of the RCA-CACHET signal detection paradigm is the use of fluorophore combinations designed for multiparametric colour coding[15], thereby markedly increasing the number of targets that could be analysed simultaneously.

RCA shows promise as a reporter system for padlock probes bound to naked DNA fibers on solid surfaces, and should be extensible to *in situ* probing with multiple probes using RCA-CACHET. Detection of padlock probes by RCA works with variable efficiency in partially deproteinized cytological preparations such as salt-extracted halo preps, but not as well in intact nuclei. These observations suggest that the extent of chromatin condensation and the fixation methods used for cytological preparations influence the efficiency of the RCA reaction. Thus, the optimization of the RCA method for allele discrimination *in situ* of single-copy genes will require further work. Nonetheless, the absence of nonspecific signals in the solution HRCA assays, together with the observation that the *in situ* RCA signals are generally limited to one or two fluorescent dots per nucleus, supports the idea that padlock probes amplified by RCA will provide sufficient sensitivity and specificity for the cytological detection of single-copy genes in the context of the total human genome.

Methods

DNA oligonucleotides. Oligonucleotides containing a phosphate group at the 5′ end were purchased from the Yale University Critical Technologies facility. The sequences are: *CFTR G542X* phosphorylated open circle probe (85 bases) for the gap oligo method, 5′–AAGAACTATATTGTCTTTCATTCTTGCATG-GTCACACGTCGTTCTAGTACGCTTCTAGTACGCTTTTCCACTCAGTG-TGATTCCA–3′; *G542X* wild type phosphorylated gap probe, 5′–CCTTCTCC–3′; *G542X* mutant phosphorylated gap probe, 5′–CCTTCTCA–3′; primer for rolling circle reaction, 542X.P1.18, 5′–ACGACGTGTGACCATGCA–3′. For the polymerase gap-fill method, the phosphorylated open circle probe (89 bases) was 5′–GAACTATATTGTCTT-TCTCTGATTCTGACTCGTCATGTCTCAGCTTTAGTTTAATACGACTCA CTATAGGGCTCAGTGTGATTCCACCT–3′. Primers for the HRCA reactions performed with gap-fill probe were: first primer, 542X.P1-23, 5′–CTAAAGCTGAGACATGACGAGTC–3′; alternative second primers for allele discrimination, 542X.P2-23-C, 5′–CTCAGTGTGATTCCACCTTC–3′ or 542X.P2-23-A, 5′–CTCAGTGTGATTCCACCTTCACA–3′; *G542X* artificial wild type target, 5′–TTGCAGAGAAAGACAATATAGTTCTTGGAGAAG-GTGGAATCACACTGAGTGGA–3′; *G542X* artificial mutant target, 5′–TTGCAGAGAAAGACAATATAGTTCTTTGAGAAGGTGGAATCACACTG-AGTGGA–3′. Phosphorylated open circle probe specific for base number 114 of intron 9 of *OCT*: 5′–GAGGAGAATAAAAGTTTCTCATAAGACTCG-TCATGTCTCAGCAGCTTCTAACGGTCACTAATACGACTCACTATAGG-TTCTGCCTCTGGGAACAC–3′. *OTC* wild-type gap probe was 5′–TAGT-GATC–3′. Primer for RCA, OTC.P.18, 5′–GCTGAGACATGACGAGTC–3′. Oligonucleotides for the RCA-CACHET method, carbon spacers in parenthesis: phosphorylated P1-G542X, 5′–GAGAAGGTGGAATCACACTGAGTG-GAGGTCAACGAGCAATTTTTTTTTTT-(C7-NH2)–3′; P2wt, 3′–GTT-CTTGATATAACAGAAAGTTTT-(C18)-TTTTTATGATCACAGCTGAGGA-TAGGACATGCGA–3′; P2mu, 3′–TTTCTTGATATAACAGAAAGTTTT-(C18)-TTTTTACGTCGTCCGTGCTAGAAGGAAACACGCA–3′; pre-made amplification circles with cognate detector tags, Cwt, 5′–CGCATGTCCTAT-CCTCAGCTGTGATCATCAGAACTCACCTGTTAGACGCCACCAGCTCC-AACTGTGAAGATCGCTTAT–3′; detector tag Fl-det1c-dinitrophenol (DNP), FITC-TCAGAACTCACCTGTTAG-3′-DNP; detector tag Fl-det1d-DNP, FITC-ACTGTGAAGATCGCTTAT–3′-DNP; Cmu, 5′–GCGTGTTTC-CTTCTAGCACGGACGACGTATATGATGGTACCGCAGCCAGCATCACC-AGACTGAGTATCTCCTATCACT–3′; detector tag Cy3-det2b-DNP, Cy3-TATATGATGGTACCGCAG–3′-DNP; detector tag Cy3-det2c-DNP, Cy3-TGAGTATCTCCTATCACT–3′-DNP.

Enzymes, antibodies and cell lines. Phage ø29 DNA polymerase was a gift from M. Salas and B. Moffett. *T. thermophilus* DNA ligase was a gift from F. Barany. Some ligation reactions were performed with Ampligase DNA (Epicentre Technologies). Other proteins and enzymes used were *T. flavus* DNA polymerase (Molecular Biology Resources), Sequenase 2.0 (Amersham), phage T4 gene 32 protein (Amersham), Vent (exo-) DNA polymerase (New England Biolabs), T7 RNA polymerase (Ambion) and biotinylated anti-bromouridine deoxyriboside (BUDR) IgG (Zymed). Anti-DNP IgM was from American Research Products. Cell lines of known genotypes for the *G542X* locus (GM07828[+/+], GM11497B[+/–], GM11496[–/–]) were purchased from Corriell Cell Repositories.

Probe ligations using gap oligonucleotides or gap-filling. Reactions employing gap oligonucleotides for the detection of artificial targets contained 150 nM open circle probe, 1300 nM gap probe, 420 nM artificial target in 20 mM Tris•HCl (pH 8.3), 50 mM KCl, 0.5 mM NAD, 10 mM $MgCl_2$ and 0.01% Triton X-100. Reactions with genomic DNA targets were performed in the same buffer, using 50 nM open circle probe and 140 nM gap oligonucleotide. *T. thermophilus* DNA ligase was used at a concentration of 0.4 Units/µl, in a 40 µl reaction. Ligation was performed for 1 h at 52 °C, which is higher than the calculated T_m for the gap oligonucleotide. The circularizable *G542X* probe and an equimolar mixture of the two allele-specific gap oligonucleotides were ligated in the presence of the mutant sequence artificial DNA target. Circular DNA resulting from the ligation of *G542X* gapped probes were amplified with ø29 DNA polymerase, and the amplified ssDNA was cloned and sequenced. The resulting sequence contained only T at the position of the *G542X* mutation in 20 independent clones, indicating that only the mutant gap oligonucleotide had been ligated and amplified. With the wild-type template, the RCA product contained only G at the *G542X* site in 20 clones (data not shown). Thus, the amplified material contained faithful replicas of a small segment of the target DNA. For combined gap-filling and ligation reactions, the open-circle probe concentration was 135 nM when artificial targets were used, and 70 nM for genomic DNA targets. The reaction was performed in 50 mM (N-[2-Hydroxyethyl]pyperazine)-N′-3-propanesulfonic acid (EPPS)-KOH pH 7.8, 25 mM $MgCl_2$, 0.25 mM NAD, 400 µM dATP, dCTP, dTTP, 0.075 U/µl *T. flavus* DNA polymerase, 0.37 U/µl Ampligase DNA ligase. Artificial targets were at a final concentration of 420 nM. For genomic DNA targets, the DNA concentration was 850 ng in 40 µl. The gap-fill reaction was incubated at 60 °C for 45 min, and 4 µl were used for analysis by HRCA. The circular oligonucleotides used two-colour RCA-CACHET were ligated using an artificial guide oligonucleotide and standard ligation conditions.

Rolling-circle reactions. Reactions contained 10 nM circularized probes, 130 nM rolling-circle primer, in 50 mM Tris•HCl (pH 7.5), 10 mM $MgCl_2$, 200 µg/ml acetylated BSA (Amersham) 400 µM dATP, dGTP, dCTP and dTTP. Phage T4 gene-32 protein was present at a concentration of 40 ng/µl (approximately 1142 nM), and phage ø29 DNA polymerase was added last at a concentration of 6.3 ng/µl (approximately 92 nM). The reactions were incubated at 31 °C for periods ranging from 2–30 min.

RCA-CACHET mutation detection assays on glass slides. The synthetic oligonucleotides used are listed above. The oligonucleotide P1-G542X was immobilized over an area of 1 mm in diameter on the surface of a glass slide activated with reactive groups. The slides were coated with 4-aminobutyl-dimethylmethoxysilane and derivatized with 1,4-phenylene-diisothiocyanate, using a published protocol[16] with minor modifications. Covalent coupling was obtained by reaction of a primary amine attached to the 3′ end of the P1-G542X oligonucleotide. Genomic DNA preparations obtained from human cell lines that were either wild type or homozygous mutant at the *CFTR-G542X* locus were mixed in different pre-determined ratios (mutant/wild type equal to 1:0, 1:1, 1:25 and 1:100) and then amplified by PCR as described[17]. PCR amplicons were used as targets for allele discrimination assays on glass slides that contained immobilized P1 molecules. The DNA was denatured at 98 °C for 5 min immediately before use. Hybridization/ligation was carried out in 7.5 µl of 20 mM Tris•Cl, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM NAD, 200 ug/ml BSA and 0.5 unit/µl of Ampligase (Epicentre). Target concentration was in the 0.1–0.2 nM range. Probe-primers P2-wt and P2-mu were 1 µM each. All enzymatic reactions took place by confining the reaction volume over the DNA dot with a small silicone rubber O-ring sealed with rubber cement. The slide was covered with a second slide, forming a sandwich filled with wet filter paper perforated at the positions of the O-rings. This sandwich was placed on an aluminum block in a pre-heated moisture chamber. Ligation took place at 62 °C for 2 h. Washes were performed at 70 °C, as follows: twice for 3 min in 75% formamide + 2× SSPE (1×SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% SDS, 1 min in 0.05% Triton X-100, 2 min with 6× SSPE, 0.05% Triton X-100, 1 min in 0.01% Triton X-100 and finally a brief rinse in water. Hybridization of signal-generating circular oligonucleotides (Cwt and Cmu, see oligonucleotide section) was performed using a concentration of 170 nM for each circle in 6 µl of Buffer 1 (67 mM Tris•Cl, pH 8.0, 16.7 mM $MgCl_2$, 84 mM NaCl, 8 mM DTT, 0.0167% Triton X-100). After 30 min at 45 °C, the slide was placed on ice, 2 µl of Buffer II (containing 20 µM *E. coli* SSB, 25 mM DTT, and 1.5 mM each of dATP, dCTP, dGTP and dTTP) was added, and incubation proceeded for 5 min at 37 °C. Two µl of 5 µM Sequenase Version 2.0 DNA polymerase was added, followed by incubation at 37 °C for 20 min. Stringent washes at 70 °C were as follows: twice for 3 min in 75% formamide, 2×SSC, 0.1% SDS (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 1 min in 0.2×SSC + 0.05% Triton X-100, 2 min in 4×SSC, 0.05% Triton X-100. Each dot was covered with 7.5 µl of detection tag oligonucleotides (0.5 µM each of Fl-det1c-DNP, Fl-det1d-DNP, Cy3-det2b-DNP,Cy3-det2c-DNP in 2×SSC + 0.05% Triton X-100 + 0.5 mg/ml degraded herring sperm DNA) and incubated at 37 °C for 20 min. The slide was washed 4 times for 5 min with 2×SSC + 0.1% Tween 20, and 4 times for 5 min with 4×SSC + 0.05% Triton X-100, rinsed once with 2×SSC and drained. 7.5 µl of condensation solution (33 µM mouse anti-DNP IgM in 2× SSC, 0.1% Tween 20, 0.5% BSA, 1 mg/ml degraded herring sperm DNA) was added, and the slide was incubated at 37 °C for 15 min and washed twice for 5 min in 2×SSC, 0.1% Tween 20 at RT, drained and covered with Pro-long antifade (Molecular Probes Inc.) under a cover slip. Fluorescent imaging was performed using a Zeiss epifluorescence microscope equipped with a Photo-

metrics cooled CCD camera. For an oligonucleotide spot of 1 mm in diameter, the maximum number of non-overlapping green or red signals is about 80,000 using our CACHET conditions. Optimization of reaction parameters may permit even higher signal densities without cross-talk.

HRCA reactions. A 4-µl aliquot of a ligation reaction was incubated in a volume of 35 µl containing 20 mM Tris•HCl (pH 8.8), 10 mM KCl, 2.7 mM $MgSO_4$, 5% v/v DMSO, 0.1% Triton X-100, 400 µM dATP, dGTP, dCTP, dTTP and 900 nM each of two specific primers (542X.P1-23, combined with either 542X.P2-23-C, or 542X.P2-23-A). Phage T4 gene-32 protein (Amersham) was present at a concentration of 38 ng/µl, (approximately 1085 nM). After combining all these materials at RT, the reactions were placed in ice, Vent (exo-) DNA polymerase (New England Biolabs) was added at a final concentration of 0.32 units/µl, and the reactions were incubated at 92 °C for 3 min, then at 65.5 °C for 90 min. The conditions described here represent experimentally determined optima for primer concentration (operational range 600–1200 nM), magnesium ion concentration (operational range 2.2–3.0 mM when using 400 µM dNTPs) and temperature (62–66.5 °C for Vent DNA polymerase, 60–65 °C for Bst DNA polymerase). Primers should have a T_m slightly higher than the HRCA reaction temperature. Four different batches of T4 Gene 32 protein have been used successfully; one of the four batches was less stimulatory than the other three, but still satisfactory for exponential amplification (Fig. 5*c*).

In situ detection of padlock probes by RCA. Human DNA was bound on polylysine slides as described[18]. Human peripheral lymphocytes were processed as halo preparations as described[11,12]. Padlock probe ligation *in situ* was performed for 2 h at 52 °C by incubating 18 µl of reaction mixture under a cover slip sealed with rubber cement as follows: 150 nM phosphorylated *G542X* 89-mer probe, 1200 nM wild type *G542X* gap probe, in 20 mM Tris•HCl (pH 8.3), 50 mM KCl, 0.5 mM NAD, 10 mM $MgCl_2$, 150 µg/ml acetylated BSA, 0.01% Triton X-100 and 0.7 U/µl Ampligase DNA ligase. The slide was washed twice for 5 min at 50 °C in 2×SSC, 25% formamide, then washed for 2 min in 50 mM Tris-Cl, pH 7.5, 40 mM KOAC, 10 mM $MgCl_2$, 10 mM DTT, 100 µg/ml BSA to remove formamide. An RCA reaction was carried out in a volume of 12 µl under a coverslip using a reaction buffer consisting of 50 mM Tris•HCl (pH 7.5), 10 mM $MgCl_2$, 200 µg/ml acetylated BSA 400 µM dATP, dGTP, dCTP and dTTP and a rolling-circle primer (600 nM oligonucleotide 542X.P1.18). Phage T4 gene-32 protein was present at a concentration of 40 ng/µl (approximately 1142 nM), and phage ø29 DNA polymerase was added at a concentration of 6.3 ng/µl (approximately 92 nM). The RCA reactions were incubated at 37 °C for either 22 min (for polylysine slides) or 15 min (for halo slides). Each slide was then washed twice for 5 min in 2×SSC with 20% formamide at 25 °C, and twice for 4 min in SSC-BSAT (2×SSC, 2.8% BSA, 0.12% Tween-20) at 37 °C. Slides were incubated for 20 min at 37 °C in SSC-BSAT, containing 5 µg/ml biotinylated Anti-BUDR IgG. The slides were washed three times for 5 min in SSC-BSAT to remove excess antibody, and incubated for 30 min at 37 °C in SSC-BSAT containing 5 µg/ml FITC-Avidin. After three washes in SSC-BSAT, the slides were stained for 2 min in 2×SSC, 0.1 µg/ml 4´6-diamidino-2-phenlindole, washed for 10 min in 1.5×SSC, 0.01% TWEEN-20 at RT and prepared for imaging by covering with 20 µl of antifade under a cover slip.

GenBank accession number. *OCT*, HUMOTC09.

Acknowledgements

We are indebted to M. Salas for a generous gift of ø29 DNA polymerase, and for advice regarding the use of the enzyme. We also thank B. Moffett and Amersham for research samples of this enzyme. F. Barany kindly provided samples of T. thermophilus DNA ligase. C. Radding and his group kindly provided us with E. coli SSB. We thank E. Winn-Deen for critical reading of the manuscript. This work was supported in part by a research grant from ONCOR, Inc., to P.M.L. and a grant from the National Institutes of Health to D.C.W. (HG00272).

1. Nilsson, M. *et al*. Padlock probes: Circularizing oligonucleotides for localized DNA detection. *Science* **265**, 2085–2088 (1994).
2. Nilsson, M. *et al*. Padlock probes reveal single-nucleotide differences, parent of origin and *in situ* distribution of centromeric sequences in human chromosomes 13 and 21. *Nature Genet*. **16**, 252–255 (1997).
3. Fire, A. & Xu, S.Q. Rolling replication of short DNA circles. *Proc. Natl Acad. Sci. USA* **92**, 4641–4645 (1995).
4. Liu, D., Daubendiek, S. L., Zillman, M. A., Ryan, K. & Kool, E. T. Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerases. *J. Am. Chem. Soc.* **118**, 1587–1594 (1996).
5. Blanco, L. & Salas, M. Characterization and purification of a phage ø29-encoded DNA polymerase required for the initiation of replication. *Proc. Natl Acad. Sci. USA* **81**, 5325–5329 (1984).
6. Blanco, L. *et al*. Highly efficient DNA synthesis by the phage ø29 DNA polymerase. *J. Biol. Chem.* **264**, 8935–8940 (1989).
7. Abravaya, K., Carrino, J.J., Muldoon, S. & Lee, H. Detection of point mutations with a modified ligase chain reaction. *Nucleic Acids Res.* **23**, 675–682 (1995).
8. Schwarz, K. Improved yields of long PCR products using gene 32 protein. *Nucleic Acids Res.* **18**, 1079 (1990).
9. Newton, C.R. *et al*. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17**, 2503–2516 (1989).
10. Luo, J., Bergstrom, D.E. & Barany, F. Improving the fidelity of *Thermus thermophilus* DNA ligase. *Nucleic Acids Res.* **24**, 3071–3078 (1996).
11. Vogelstein, B., Pardoll, D.M. & Coffey, D.S. Supercoiled loops and eucaryotic DNA replication. *Cell* **22**, 79–85 (1980).
12. Gerdes, M.G, Carter, K.C., Moen, P.T. Jr & Lawrence, J.B. Dynamic changes in the higher-level chromatin organization of specific sequences revealed by *in situ* hybridization in nuclear halos. *J. Cell Biol.* **126**, 289–304 (1994).
13. Aliotta, J.M. *et al*. Thermostable Bst DNA polymerase lacks a 3´-5´ proofreading exonuclease activity. *Genet. Anal.* **12**, 185–195 (1996).
14. Kong, H., Kucera, R. & Jack, W. Characterization of a DNA polymerase from the hyperthermophile Archea *Thermococcus litoralis*. *J. Biol. Chem.* **268**, 1965–1975 (1993).
15. Speicher, M.R., Ballard, S.G. & Ward, D.C. Karyotyping human chromosomes by combinatorial multicolor FISH. *Nature Genet*. **12**, 368–375 (1996).
16. Guo, Z., Guifoyle, R.A., Thiel, A.J., Wang, R. & Smith, L.M. Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Res.* **22**, 5456–5465 (1994).
17. Heinoven, P., Itia, A., Torresani, T. & Lovgren, T. Simple triple-label detection of seven cystic fibrosis mutations by time-resolved fluorometry. *Clin. Chem.* **43**, 1142–1150 (1997).
18. Schena, M., Shalon, D., Davis, R.W. & Brown, P. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467–470 (1995).
19. Rychlik, W. & Rhoads, R.E. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing, and *in vitro* amplification of DNA. *Nucleic Acids Res.* **17**, 8543–8551 (1989).