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Genetics

Genetic disease detection and DNA amplification using cloned thermostable ligase

(β-globin gene/ligase chain reaction/sickle-cell allele/single-base mutation)

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ABSTRACT **Polymerase chain reaction, using thermostable DNA polymerase, has revolutionized DNA diagnostics. Another thermostable enzyme, DNA ligase, is harnessed in the assay reported here that both amplifies DNA and discriminates a single-base substitution. This cloned enzyme specifically links two adjacent oligonucleotides when hybridized at 65°C to a complementary target only when the nucleotides are perfectly base-paired at the junction. Oligonucleotide products are exponentially amplified by thermal cycling of the ligation reaction in the presence of a second set of adjacent oligonucleotides, complementary to the first set and the target. A single-base mismatch prevents ligation/amplification and is thus distinguished. This method was exploited to detect 200 target molecules as well as to discriminate between normal β^A- and sickle β^S-globin genotypes from 10-μl blood samples.**

DNA diagnostics uses the tools of molecular biology to identify nucleotide substitutions, deletions, or insertions in genes of medical interest (1). A reliable DNA diagnostics method will require faithful amplification of target sequences, accurate single-base discrimination, low background, and, ultimately, complete automation. The initial target nucleic acid amplification may be accomplished by using the polymerase chain reaction (PCR) (2), self-sustained sequence replication (3), or ligase amplification reaction (4, 5). Subsequently, single-base mismatches may be detected via allele-specific and reverse oligonucleotide hybridization (6, 7), denaturing gradient gel electrophoresis (8), RNase or chemical cleavage of mismatched heteroduplexes (9, 10), use of nucleotide analogs (11), or fluorescence PCR amplification/detection (12).

Landegren *et al.* (13) have pioneered an oligonucleotide ligation assay to circumvent the need for electrophoresis or precise hybridization conditions. Two oligonucleotide probes are hybridized to denatured DNA, such that the 3′ end of the first one is immediately adjacent to the 5′ end of the second probe. DNA ligase can covalently link these two oligonucleotides, provided that the nucleotides at the junction are perfectly base-paired to the target (4, 5, 13, 14). A single-nucleotide substitution can, therefore, be distinguished. Use of biotin on the first probe and a suitable nonisotopic reporter group on the second probe allows for product capture and detection (13) in a manner amenable to automation.

Ideally, the oligonucleotides should be sufficiently long (20–25 nucleotides) so that each will preferentially hybridize to its unique position on the human genome. The specificity of ligation should be particularly enhanced by performing the reaction at or near the melting temperature (t_m) of the two oligonucleotides. At higher temperatures a single-base mismatch at the junction forms not only an imperfect double helix but also destabilizes hybridization of the mismatched oligonucleotide.

This report describes DNA detection that uses a thermostable ligase to exquisitely discriminate between a mismatched and complementary DNA helix (Fig. 1 *Upper*). Because the enzyme retains activity after multiple thermal cycles, the ligations may be repeated to linearly increase product [termed ligase detection reaction (LDR)]. Product may be further amplified in a ligase chain reaction (LCR) by using both strands of genomic DNA as targets for oligonucleotide hybridization. Two sets of adjacent oligonucleotides, complementary to each target strand, are used. The ligation products from one round can become the targets for the next round of ligation (Fig. 1 *Upper*). By use of LCR, the amount of product can be increased in an exponential fashion by repeated thermal cycling.

MATERIALS AND METHODS

Thermostable Ligase. Plasmid libraries of *Thermus aquaticus* strain HB8 DNA (ATCC27634) were screened for the ability to complement a temperature-sensitive *lig*ts7 derivative of *Escherichia coli* [unpublished work; ref. 16]. One complementing plasmid (pDZ1) contained a thermostable ligase gene as evidenced by (*i*) presence of a thermostable NAD^+-dependent nick-closing (ligase) activity in crude extracts when assayed at 65°C (17) and (*ii*) DNA sequence analysis of the first 60 codons of the putative gene revealed >50% amino acid identity to *E. coli* ligase (18). Thermostable ligase was purified from *E. coli* cells containing the ligase gene cloned downstream of an inducible T7 expression system (19), as described elsewhere (unpublished work). Ligase activity was assayed for the ability to seal nicked plasmid DNA (pUC4KIXX) as monitored by electrophoresis on 1% agarose gel. One nick-closing unit of ligase is defined as the amount of ligase that circularizes 0.5 μg of nicked pUC4KIXX DNA in 20 μl of 20 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $MgCl_2$/1 mM EDTA/10 mM NAD^+/10 mM dithiothreitol overlaid with a drop of mineral oil after 15-min incubation at 65°C.

Genomic DNA, Plasmid DNA, and Oligonucleotides. Human genomic DNA was isolated from 0.5 ml of whole blood as described (20). Proteinase K and RNase A were removed by sequential extractions with phenol, phenol/chloroform, chloroform, 1-butanol (twice), and nucleic acid was recovered by precipitation with ethanol. Samples were boiled for 5 min before use in LCR assays. Plasmid DNAs containing the β^A- and β^S-globin gene alleles were a gift from D. Nickerson (California Institute of Technology, Pasadena, CA) and were digested with *Taq* I before use as target DNA. Oligonucleotides were assembled by the phosphoramidite method (21) on an Applied Biosystems model 380A DNA synthesizer, purified by reversed-phase HPLC, and provided

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Abbreviations: PCR, polymerase chain reaction; LDR, ligase detection reaction; LCR, ligase chain reaction.

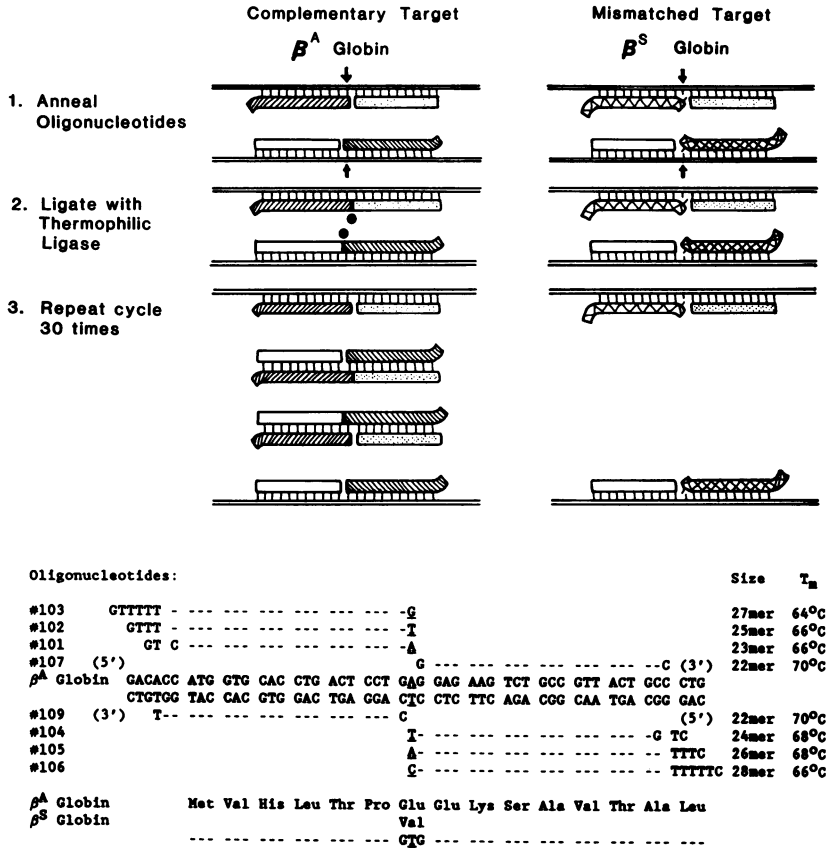

FIG. 1. (*Upper*) Diagram depicting DNA amplification/detection by using LCR. DNA is heat denatured, and four complementary oligonucleotides are hybridized to the target at a temperature near their melting temperature (65°C; t_m). Thermostable ligase will covalently attach only adjacent oligonucleotides that are perfectly complementary to the target (*Left*). Products from one round of ligations become targets for the next round, and thus products increase exponentially. Oligonucleotides containing a single-base mismatch at the junction do not ligate efficiently and, therefore, do not amplify product (*Right*). (*Lower*) Nucleotide sequence and corresponding translated sequence of the oligonucleotides used in detecting β^A- and β^S-globin genes. Oligonucleotides 101 and 104 detect the β^A target, whereas oligonucleotides 102 and 105 detect the β^S target when ligated to labeled oligonucleotides 107 and 109, respectively. Oligonucleotides 103 and 106 were designed to assay the efficiency of ligation of G–T or G–A and C–A or C–T mismatches when using β^A- or β^S-globin gene targets, respectively. Oligonucleotides have calculated t_m values of 66–70°C (15), just at or slightly above ligation temperature. The diagnostic oligonucleotides (101–106) contained slightly different length tails to facilitate discrimination of various products when separated on polyacrylamide denaturing gel.

by R. Kaiser and S. Horvath (California Institute of Technology, Pasadena, CA). Oligonucleotide sequences (5′–3′) are: 101, GTCATGGTGCACCTGACTCCTGA; 102, GTTTCATGGTGCACCTGACTCCTGT; 103, GTTTTTCATGGTGCACCTGACTCCTGG; 104, CTGCAGTAACGGCAGACTTCTCCT; 105, CTTTGCAGTAACGGCAGACTTCTCCA; 106, CTTTTTGCAGTAACGGCAGACTTCTCCC; 107, GGAGAAGTCTGCCGTTACTGCC; 109, CAGGAGTCAGGTGCACCATGGT. (See Fig. 1.)

^{32}P Labeling of Oligonucleotides. Oligonucleotides 107 or 109 (0.1 μg = 15 pmol) were 5′ end-labeled in 20 μl of 30 mM Tris·HCl, pH 8.0/20 mM Tricine/10 mM $MgCl_2$/0.5 mM EDTA/5 mM dithiothreitol/400 μCi of [γ-^{32}P]ATP (6,000 Ci/mM = 60 pmol ATP, New England Nuclear; 1 Ci = 37 GBq) by addition of 15 units of T4 polynucleotide kinase (New England Biolabs). After incubation at 37°C for 45 min, unlabeled ATP was added to 1 mM, and incubation was continued an additional 2 min at 37°C. The reaction was terminated by adding 0.5 μl of 0.5 M EDTA, and the kinase was heat-inactivated (65°C for 10 min). Unincorporated ^{32}P label was removed by chromatography with Sephadex G-25 preequilibrated with Tris/EDTA buffer. Specific activity ranged from 7 to 10 × 10^8 cpm/μg of oligonucleotide.

LDR and LCR Reaction Conditions. For LDR reactions, labeled oligonucleotide (200,000 cpm = 0.28 ng = 40 fmol) and unlabeled diagnostic oligonucleotide (0.27 ng = 40 fmol) were incubated in the presence of target DNA (1 fmol = 6 × 10^8 molecules of *Taq* I-digested β^A- or β^S-globin plasmid) in 10 μl of 20 mM Tris·HCl, pH 7.6/100 mM KCl/10 mM $MgCl_2$/1 mM EDTA/10 mM NAD^+/10 mM dithiothreitol/4 μg of salmon sperm DNA/15 nick-closing units of *T. aquaticus* ligase and overlaid with a drop of mineral oil. Reactions were incubated at 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated 5 or 20 times. For LCR reactions, unlabeled diagnostic oligonucleotide pairs (101 and 104, 102 and 105, or 103 and 106; 40 fmol each) and adjacent pairs of labeled oligonucleotides (107 and 109, 40 fmol each) were incubated in the presence of ligase and target DNA (ranging from 100 amol to less than one molecule per tube) with 20 or 30 cycles as described above.

Electrophoresis. Samples (4 μl) were in 45% formamide and denatured by boiling for 3 min before loading (40,000 or 80,000 cpm/lane). Electrophoresis was in 10% polyacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate, pH 8.9/1 mM EDTA for 2 hr at 60-W constant power. After removing urea, gels were dried and autoradiographed overnight at −70°C on Kodak XAR-5 film with the aid of a Cronex intensifying screen (DuPont).

RESULTS

The gene encoding human β-globin was selected as a model system to test ligation amplification and detection. The normal β^A and sickle β^S genes differ by a single A → T transversion that leads to a change of a glutamic acid residue to a valine in the hemoglobin β chain [Fig. 1, *Lower* (22)]. Diagnostic oligonucleotides containing the 3′ nucleotide unique to each allele were synthesized with different-length 5′ tails (Fig. 1 *Lower*). Upon ligation to the invariant ^{32}P-labeled adjacent oligonucleotide, the individual products could be distinguished when separated on a polyacrylamide denaturing gel and detected by autoradiography.

Specificity of Thermostable Ligase. The specificity of ligating oligonucleotide pairs on a target DNA with perfect complementarity was directly compared with each possible mismatch (see Fig. 2 and Table 1). Results show that *T. aquaticus* ligase efficiently links correctly base-paired oligonucleotides and gives near zero ligation in the presence of a mismatch (Table 1). When only 1 fmol of target DNA was used under LDR conditions, the worst mismatches were 1.5–1% (G–T, T–T), whereas other mismatches were <0.4% (A–A, C–T, G–A, G–A) of the products formed with complementary oligonucleotide base pairs (A·T). This is substan-

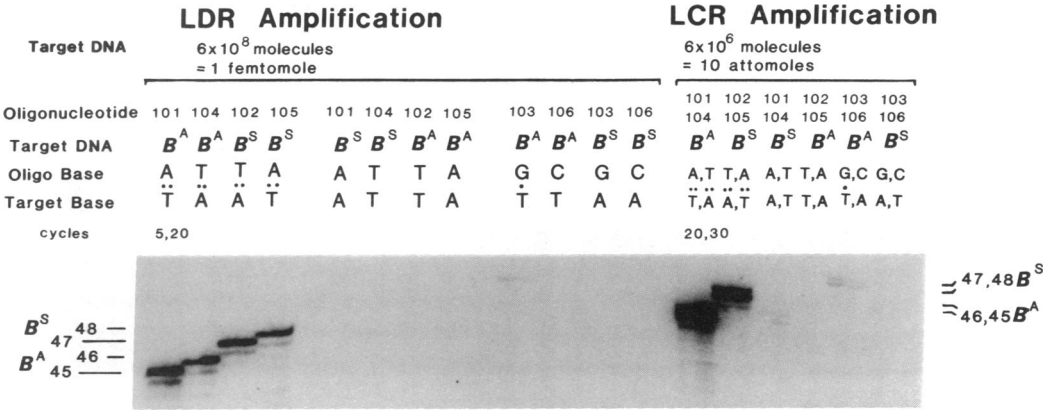

FIG. 2. Autoradiogram showing specificity of *T. aquaticus* ligase under LDR and LCR amplification conditions. Specificity was assayed by ligation of diagnostic oligonucleotides in the presence of either complementary or mismatched β^A- or β^S-globin gene target DNA (LDR amplification). Ligation of diagnostic oligonucleotides 101 (β^A allele), 102 (β^S allele), or 103 to labeled 107 gives lengths of 45, 47, or 49 nucleotides, respectively. For the complementary strand, ligation of diagnostic oligonucleotides 104 (β^A allele), 105 (β^S allele), or 106 to labeled 109 gives lengths of 46, 48, or 50 nucleotides, respectively. The diagnostic oligonucleotide listed in each lane and the appropriate adjacent labeled oligonucleotide (40 fmol each) was incubated with target DNA (1 fmol = 6×10^8 molecules of *Taq* I-digested β^A- or β^S-globin plasmid), as described. In LCR amplification, samples contained pairs of diagnostic oligonucleotides (β^A allele-specific 101 and 104, β^S allele-specific 102 and 105, or "C–G pair" 103 and 106), both labeled oligonucleotides (107 and 109), and were incubated with ligase and 10 amol of target DNA (6×10^6 molecules; 100-fold less than for LDR) as described. Samples were loaded in groups of eight and run into the gel; then the next set was loaded. This accounts for the "slower" migration of bands on the right side of the autoradiogram. (Intensifying screen was not used for this autoradiogram.) Bands were excised from the gel and assayed for radioactivity (Table 1).

tially better than found for mesophilic T4 or *E. coli* ligase when using similar radioactive detection methods (13, 14).

In the amplification/detection (LCR) experiments, four oligonucleotides were incubated with ligase and 10 amol of target DNA (see Fig. 2 *Right* and Table 1 lower part). The 3′ nucleotide of each unlabeled diagnostic oligonucleotide was either complementary or mismatched to the target DNA and yet was always complementary to its pair—i.e., A·T for 101 and 104, T·A for 102 and 105, and G·C for 103 and 106.

Table 1. Quantitation of complementary and mismatched LDR and LCR

Amplification	Oligonucleotide base–target base	Product formed, %*	Mismatched/ complementary, %†
LDR (6×10^8 target molecules = 1 fmol)	A–T	21.5	
	T–A	13.2	
	T–A	17.9	
	A–T	12.4	
	A–A	<0.1	<0.4
	T–T	0.12	0.7
	T–T	0.16	1.0
	A–A	<0.1	<0.4
	G–T	0.30	1.4
	C–T	<0.1	<0.4
	G–A	<0.1	<0.4
	C–A	<0.1	<0.4
LCR (6×10^6 target molecules = 10 amol)	A–T, T–A	41.4	
	T–A, A–T	10.4	
	A–A, T–T	0.45	1.1
	T–T, A–A	<0.05	<0.2
	G–T, C–A	0.51	1.3
	G–A, C–T	<0.05	<0.2

Bands from 20-cycle LDR and 30-cycle LCR experiments described in Fig. 2 were excised from the gels and assayed for radioactivity.

*Percentage product formed = cpm in product band/cpm in starting oligonucleotide band.

†Percentage mismatched/complementary = cpm in band of mismatched oligonucleotide/cpm in band of complementary oligonucleotide when using the same target DNA and indicates noise-to-signal ratio.

Four-way (target independent) ligation was minimized by use of (*i*) carrier salmon sperm DNA and (*ii*) oligonucleotides designed to create single-base 3′ overhangs (this work, see Fig. 1) or single-base 5′ overhangs (not tested). Note that an initial "incorrect" ligation of a mismatched oligonucleotide to target DNA would subsequently be amplified with the same efficiency as a correct ligation (See Fig. 1). Nevertheless, the worst mismatches were 1.3% to 0.6% (G–T, C–A; A–A, T–T), whereas others were <0.2% (T–T, A–A; G–A, C–T) of the products formed with complementary basepairs (A·T, T·A). LCR, using thermostable ligase, is thus the only method that can both amplify and detect single-base mismatches with high signal-to-noise ratios (4, 5).

The entire set of experiments described above was repeated with a buffer containing 150 mM instead of 100 mM KCl. Results were essentially the same as in Fig. 2 and Table 1; mismatches for LDR ranged from 0.6% to <0.3% and for LCR ranged from 1.7% to <0.3% of the complementary products (data not shown). Thus for *T. aquaticus* ligase, discrimination between matched and mismatched oligonucleotides is not critically dependent on salt conditions, in contrast to the requirements for mesophilic ligases (4, 5, 13, 14).

Specificity of LCR DNA Amplification with Sub-amol Quantities of Target DNA. The extent of LCR DNA amplification was determined in the presence of target DNA ranging from 100 amol = 6×10^7 molecules to <1 molecule per tube (Fig. 3, Table 2). In the absence of target DNA, no background signal was detected when carrier salmon sperm DNA (4 μg) was present (compare last 8 lanes of Fig. 3). At higher target concentration, DNA amplification was essentially complete after 20 cycles, whereas at lower initial target concentration substantially more product is formed with additional amplification cycles. After 30 cycles of LCR, 200 molecules of initial target DNA were amplified 1.7×10^5 fold and thus could be readily detected. The average efficiency of ligation per cycle (40–50%, calculated as described in ref. 4) could be potentially enhanced by altering buffer conditions [such as using NH_4Cl, $MnCl_2$, polyamines, or polyethylene glycols (17)], enzyme concentration, or thermal-cycling times and temperatures.

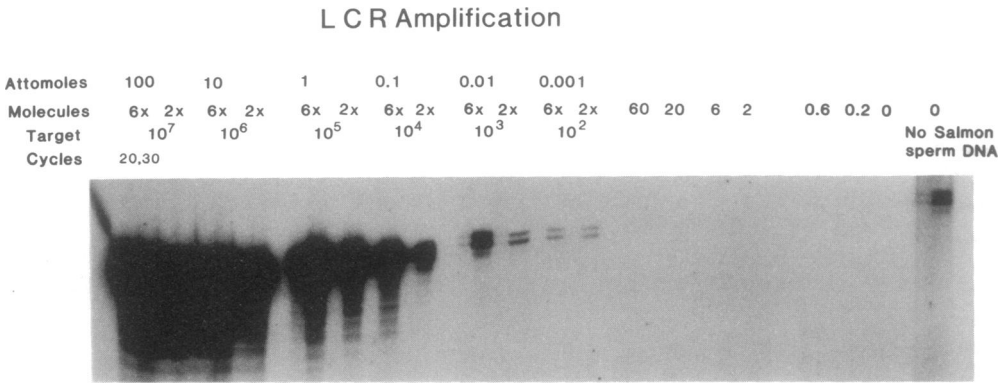

FIG. 3. Autoradiogram showing LCR amplification at different target concentrations. Labeled invariant oligonucleotides (107 and 109; 200,000 cpm = 40 fmol each) and unlabeled β^A allele oligonucleotides (101 and 104; 40 fmol each) were incubated with target DNA (ranging from 100 amol = 6×10^7 molecules to <1 molecule per tube of *Taq* I-digested β^A-globin plasmid) and ligase as described. Samples were electrophoresed, gel was autoradiographed overnight, and bands were counted as described (see Table 2). Bands of 45 and 46 nucleotides correspond to ligation products of the coding and complementary β^A-globin oligonucleotides. Lower-molecular-mass products correspond to ligation of minor species in the synthesized oligonucleotide preparations that were shorter than intended product. Samples were loaded in groups of eight, giving the appearance of slower migration on the right of the autoradiogram.

To test ligase discrimination between complementary and mismatched oligonucleotides in a direct competition assay, the above LCR experiment was repeated with or without oligonucleotides that would give G–T and C–A mismatches (see Table 3). At higher target concentrations, the mismatched product ranged from 1.8% to 0.5% of the complementary product. Mismatched product could not be detected when using <3 amol of target DNA. As control, excess mismatched target DNA (β^S- instead of β^A-globin DNA at 6×10^7 molecules per tube) gave only 2.1% and 1.5% product. Thus, the signal from the correctly paired ligation products is 50- to 500-fold higher than from mismatched products, under either competition or individual LCR ligation conditions.

Detection of β-Globin Alleles in Human Genomic DNA. DNA isolated from the blood of normal ($\beta^A\beta^A$), carrier ($\beta^A\beta^S$), and sickle cell ($\beta^S\beta^S$) individuals was tested for allele-specific LCR detection. With target DNA corresponding to 10 μl of blood, β^A and β^S alleles could be readily detected by using allele-specific LCR (Fig. 4). As seen with plasmid-derived target DNA (see Fig. 2), efficiency of ligation (and hence detection) is somewhat less for β^S- than β^A-specific oligonucleotides. This difference may be a function of the exact nucleotide sequence at the ligation junction or the particular oligonucleotides (with differing 5′ tails) used in these LCR experiments. Nevertheless, the results show the feasibility of direct LCR allelic detection from blood samples without any need for primary PCR or self-sustained sequence replication amplification.

Table 2. Quantitation of LCR amplification

Target molecules	Product formed, %*	Amplification†
6×10^7	134‡	
2×10^7	96	
6×10^6	107‡	
2×10^6	78	
6×10^5	85	
2×10^5	48	5.8×10^4
6×10^4	25	1.0×10^5
2×10^4	4.5	5.4×10^4
6×10^3	2.3	9.2×10^4
2×10^3	0.36	4.3×10^4
6×10^2	0.18	7.2×10^4
2×10^2	0.14	1.7×10^5
$60 \rightarrow 0$§	<0.05	

Bands from 30-cycle LCR experiment described in Fig. 3 were excised from gels and assayed for radioactivity.
*Percentage product formed = cpm in product band/cpm in starting oligonucleotide band.
†Amplification = no. of product molecules formed/no. of target molecules.
‡At higher target concentration, DNA amplification was essentially complete after 20 cycles; slightly imprecise excision of 30-cycle bands from this portion of the gel probably accounts for product formed values >100%.
§Product formed from 0 to 60 target molecules was indistinguishable from background (see Fig. 3).

DISCUSSION

The specificity, yield, and sensitivity of PCR were significantly improved by incorporating use of a thermostable DNA polymerase (2), resulting in a simplified procedure that has

Table 3. Quantitation of LCR amplification with or without mismatched competitor oligonucleotide

	Complementary oligonucleotides	Complementary and mismatched oligonucleotides	
Target molecules	Product formed, %*	Product formed, %*	Mismatched/ complementary, %†
6×10^7 (β^A)	114*	93	1.0
2×10^7 (β^A)	93	95	1.8
6×10^6 (β^A)	102*	93	0.5
2×10^6 (β^A)	90	67	0.5
6×10^5 (β^A)	51	46	
2×10^5 (β^A)	31	23	
6×10^4 (β^A)	17	9.3	
2×10^4 (β^A)	8.6	2.9	
6×10^3 (β^A)	3.2	0.8	
0	<0.1	<0.1	
6×10^7 (β^S)	2.1	1.5	

One set of experiments contained 40 fmol each of β^A allele oligonucleotides 101 and 104 per tube, exactly as described for Fig. 3, whereas the second set had, in addition, 40 fmol each of oligonucleotides 103 and 106 per tube (forming G–T and C–A mismatches, respectively). Bands from 30-cycle LCR experiment, as described for Fig. 3, were excised from the gels and assayed for radioactivity.
*Percentage product formed = cpm in complementary product band/cpm in starting oligonucleotide band. Imprecise excision of two bands from the gel probably accounts for product formed values >100% (see Table 2).
†Percentage mismatched/complementary = cpm in bands of mismatched oligonucleotide products/cpm in band of complementary oligonucleotide products in same lane and indicates noise-to-signal ratio.

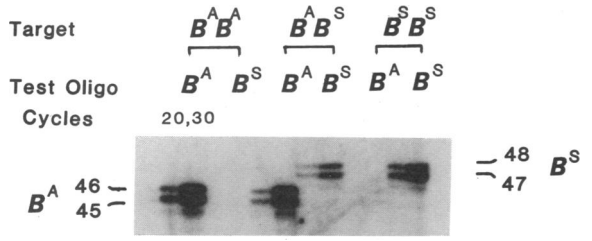

FIG. 4. Detection of β-globin alleles in human genomic DNA by autoradiogram. DNA was isolated from blood samples of normal ($\beta^A\beta^A$), carrier ($\beta^A\beta^S$), and sickle cell ($\beta^S\beta^S$) individuals as described. Genomic DNA (corresponding to 10 μl of blood or ≈ 6×10^4 nucleated cells) was tested in two separate tubes containing labeled oligonucleotides (107 and 109; 200,000 cpm = 40 fmol each) and either unlabeled β^A test oligonucleotides (101 and 104) or unlabeled β^S test oligonucleotides (102 and 105; 40 fmol each). Both reaction mixtures were incubated under the same buffer (without salmon sperm DNA), enzyme, and cycle conditions described. Samples were electrophoresed, and the gel was autoradiographed overnight as described. Ligation products of 45 and 46 or 47 and 48 nucleotides indicate presence of the β^A- or β^S-globin gene, respectively. Oligo, oligonucleotide.

become widely applicable (23, 24). Similarly, this report demonstrates the utility of thermostable ligase for allelic-specific gene detection under both LDR and LCR conditions. Both LCR and PCR amplification derive their specificity from the initial hybridization of primer to target DNA, and this is enhanced by (*i*) use of oligonucleotides of sufficient length to be unique in the human genome and (*ii*) use of temperatures near the oligonucleotide t_m. LCR amplification faithfully detected as few as 200 initial target molecules, as well as both β^A and β^S alleles directly from genomic DNA. LCR did not amplify a T–T, G–T, C–T, or C–A 3′-terminal mismatch, as has been reported for allele-specific PCR amplifications (25). Whether LCR will tolerate internal mismatches present in viral variants remains to be determined (25).

LCR amplification/detection is compatible with a primary amplification of genomic DNA by either PCR (2) or self-sustained sequence replication (3). Such a primary amplification could allow for LCR detection of emerging viral subpopulations where the mutations are known, such as the multiple mutations in human immunodeficiency virus conferring resistance to 3′-azido-3′-deoxythymidine (AZT) (26). One can also envisage multiplexing the primary amplification of dozens of loci simultaneously (27) and aliquoting products into separate microtiter wells. A subsequent round of LCR amplification/detection could then distinguish a particular target loci, even if it were initially amplified only in the amol range. Such a multiplex PCR/LCR detection assay, with the potential for an automated format, could (*i*) rapidly screen large populations for monogenic disease polymorphisms, (*ii*) distinguish several polymorphisms simultaneously from a single sperm to map the relative positions of these polymorphisms (28), and (*iii*) help eliminate current ambiguities in DNA identification of individuals for forensic or paternity cases (29).

The potential uses of thermostable enzymes that survive the temperature-cycling conditions required to denature double-stranded DNA are just now being tapped. With variations of the LCR concepts outlined above, thermostable ligase could be used to (*i*) covalently capture specific DNA fragments to a solid matrix, with the aid of "template oligonucleotides" (40- to 50-mers) complementary to both the fragment end as well as a second oligonucleotide attached to a solid support, (*ii*) covalently link PCR-generated fragments (for example, protein domains or exons) in specific order, and (*iii*) covalently link two members of a hexamer oligonucleotide library to form specific dodecamers for directed sequencing of cosmids and other large DNAs (30).

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