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Peptide Nucleic Acid Array for Detection of Point Mutations in Hepatitis B Virus Associated with Antiviral Resistance▽†

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The detection of antiviral-resistant hepatitis B virus (HBV) mutations is important for monitoring the response to treatment and for effective treatment decisions. We have developed an array using peptide nucleic acid (PNA) probes to detect point mutations in HBV associated with antiviral resistance. PNA probes were designed to detect mutations associated with resistance to lamivudine, adefovir, and entecavir. The PNA array assay was sensitive enough to detect 10^2 copies/ml. The PNA array assay was able to detect mutants present in more than 5% of the virus population when the total HBV DNA concentration was greater than 10^4 copies/ml. We analyzed a total of 68 clinical samples by this assay and validated its usefulness by comparing results to those of the sequencing method. The PNA array correctly identified viral mutants and has high concordance (98.3%) with direct sequencing in detecting antiviral-resistant mutations. Our results showed that the PNA array is a rapid, sensitive, and easily applicable assay for the detection of antiviral-resistant mutation in HBV. Thus, the PNA array is a useful and powerful diagnostic tool for the detection of point mutations or polymorphisms.

Antiviral therapy in patients with chronic hepatitis B is associated with improved outcomes. Several oral agents have been approved by the U.S. Food and Drug Administration for the therapy of chronic hepatitis B, including lamivudine (LMV), adefovir (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TFV). However, hepatitis B virus (HBV) mutations associated with antiviral resistance are a major problem in the treatment of chronic hepatitis B. Antiviral resistance and poor adherence are the most important factors in the treatment failure of nucleoside and nucleotide analogue therapies for hepatitis B (8, 12, 16, 20, 25). The sensitive and early detection of emerging resistance is important for monitoring the viral dynamics associated with treatment and to improve therapeutic decision making. Consequently, the method for the detection of antiviral-resistant HBV must be rapid, sensitive, and accurate for reliable diagnosis (13, 21).

Various methods have been proposed for the detection of antiviral-resistant HBV mutants. Direct sequencing can identify known and potential new resistance mutations, but it is a time-consuming and laborious method. Furthermore, sequencing can detect minor populations comprising more than 20% of the total virus population (8, 13, 26). In particular, the sequencing method is able to correctly determine the nucleotide mixture at position 1 (R = A or G) and position 3 (K = T or G, R = A or G, and S = C or G) of codon 204 (11, 23). Thus, the mutant mixture (M204V/M204I) of codon 204 is difficult to correctly determine as the mixture of mutants and the wild type (M204V/M204I/M204) by direct sequencing. Other methods have been used to overcome some of the limitations of sequencing, including PCR restriction fragment length polymorphism (RFLP) analysis and reverse hybridization (line probe assay and oligonucleotide array). However, these methods have disadvantages in terms of specificity, sensitivity, operational complexity, and interpretation of results (1, 8, 13, 17). Most hybridization methods usually make use of a DNA probe, which can be a synthetic oligonucleotide or longer enzymatically generated DNA. Although DNA probes work well, they have problems with selectivity, sensitivity, and stability under various conditions (24). Recently, however, several nucleic acid analogs, such as peptide nucleic acids (PNAs), have been developed to overcome the limitations of natural nucleic acids for specificity, sensitivity, hybridization kinetics, thermodynamic properties, and stability (2, 15).

PNAs are a nucleic acid analog in which the negatively charged ribose-phosphate backbone is replaced by uncharged *N*-(2-aminoethyl)-glycine units. The uncharged nature of PNA is responsible for the better thermal stability of the PNA:DNA duplex compared to that of the DNA:DNA duplex. The stability of the PNA:DNA duplex is greatly affected by the presence of a single-base mismatch. A PNA:DNA mismatch is more destabilizing than a mismatch in a DNA:DNA duplex, and they therefore are highly sequence selective. The stronger binding properties and biological stability of PNA give more specific, more sensitive, and more accurate results in the detection of target sequences. These properties of PNA are widely used as a molecular tool in molecular biology and biotechnology. An increasing number of applications for PNA technology have been described, detecting specific gene sequences in advanced diagnostic methods such as PCR clamping, real-time PCR, FISH (fluorescence *in situ* hybridization), and microarrays (3, 4, 10, 24). The PNA array (for research use only, not for use in diagnostic procedures) (Panagene Inc., Daejeon, South Korea) is an *in vitro* microarray used to detect the presence of different genetic variants of HBV in human blood samples (PANArray DR HBV).

The primary aim of this study was to develop a PNA array for the detection of HBV mutations associated with antiviral

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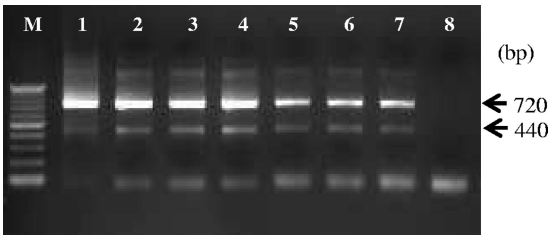

FIG. 1. Amplification of DNA template isolated from serum specimens using one-tube nested PCR. Amplified product was separated in a 2% agarose gel and shown as specific bands of 720 and 440 bp. Lane M, 100-bp DNA ladder; lanes 1 to 6, clinical isolates; lane 7, positive control (clone); lane 8, negative control.

resistance. The secondary aim was to evaluate the accuracy of the PANArray DR HBV by comparing the results of this assay to those of direct sequencing for concordance.

MATERIALS AND METHODS

Preparation of target DNA. (i) DNA extraction. Serum samples used in this study were collected from 68 patients with HBV infection who had received antiviral therapy. HBV DNA was extracted from 200-μl serum samples using the real genomics genomic DNA extraction kit (RBC Bioscience, Taipei County, Taiwan). Extracted DNA pellets were dissolved in 50 μl Tris-EDTA (TE) buffer and stored at −20°C.

(ii) PCR amplification. The target DNA was amplified in a single-tube nested PCR with three primers: two sense primers, HBM977 (5′-biotin-TGGCCAAA ATTCGCAGTCCC-3′) and HB-F3 (5′-biotin-CTTGTATTCCCATCCCATCA TC-3′), and one antisense primer, HBPr (5′-biotin-GCAAAGCCCAAAAGAC CCAC-3′). Two sense and one antisense primer were designed from nucleotides 1210 to 1229, 1487 to 1505, and 1909 to 1928 based on the polymerase gene sequence GenBank accession number AB033556. Briefly, a 50-μl reaction mixture contained 1 U of *Taq* DNA polymerase (Solgent, Daejeon, South Korea) and PCR buffer with 1.5 mM $MgCl_2$, 100 μM deoxynucleoside triphosphate mixture, and 6.25 μM each primer. A volume of 4 μl of DNA sample was added to the PCR master mix. Amplification was performed with an annealing temperature of 55°C for 35 cycles and continuously performed with an annealing temperature of 52°C for 30 cycles. The PCR products were 720 bp (HBPr977 and HBPr) and 440 bp (HB-F3 and HBPr) long (Fig. 1).

Construction of HBV clones. To verify the specificity of the probe, one clone of the wild-type and 60 clones of mutants were constructed by site-directed mutagenesis. Two mutagenic primers (sense and antisense) were designed to introduce point mutations at the center of each reference sequence, and the sequences of both sides were perfectly matched. The site-directed mutagenesis was comprised of two PCR steps. The first PCR step was performed with two tubes. One tube contained HBPr977 and the mutagenic antisense primer, and the other contained HBPr and the mutagenic sense primers. After PCR, the products were purified by using a Labopass PCR purification kit (Cosmogenetech, Seoul, South Korea). The next PCR was amplified with two purified DNA samples by HBPr977 and HBPr primers in one tube. The PCR products were purified with the Labopass PCR purification kit and cloned in vector pGEM-T (Promega, Madison, WI). To confirm the mutagenesis, we sequenced clones.

Design of PNA probe. A total of 74 specific PNA probes were designed on the basis of viral resistance-specific sequence motifs in the amplified region. The probes were designed to be suitable for the following parameters. The position of a potential point mutation was close to the center of the probe. The melting point (T_m) of PNA probes was calculated using visual OMP software (DNA Software, Inc., MI). To select the optimal PNA probes, several candidate probes for each target were designed. After optimization, these nucleotide-specific PNA probes were applied as 28 amino acid-specific probes (grouping all probes showing the same amino acid together) on a slide.

The PANArray DR HBV test covers the most important amino acid variations at 11 different codon positions, 80, 169, 173, 180, 181, 184, 202, 204, 207, 236, and 250 of HBV reverse transcriptase, which are known to be associated with LMV, ADV, and ETV (see Table S1 in the supplemental material).

Synthesis of PNA probes. PNA probes were synthesized by Panagene's technology using benzothiazole-2-sulfonyl (Bts) as an amine-protecting group (18). Several candidate probes for each target were designed to select the optimal PNA probes. The PNA probes have a 5′-terminal amine group that is necessary for the covalent attachment to an epoxy surface and have spacers that make it possible for the target to approach immobilized probes.

Fabrication of the PNA array. The PNA probes were dissolved in distilled water. The PNA probes were mixed with PANArray spotting buffer (Panagene Inc., Deajeon, South Korea). The spotting mixture was printed on epoxy-coated slides (LSEP-A; Luminano Co., Seoul, South Korea) by using a Qarray mini Microarrayer with aQu solid pins (Genetix, New Milton, United Kingdom). The probes were printed as shown in Fig. S1B in the supplemental material. The printed slides were incubated in a humidified condition (humidity was set to about 75 to 80%) for at least 9 h. The printed slide was blocked with succinic anhydride (SA) and dimethyl-formamide (DMF) at 40°C for 2 h. The slides were washed with DMF and distilled water to remove unbound probes. After being dried, the PNA array was ready for hybridization.

Hybridization and scanning. The biotin-labeled PCR fragments were denatured at 95°C for 5 min and then chilled on ice for 2 min. Five μl of biotin-labeled target DNA was mixed with 95 μl of the PNA hybridization buffer containing Cy5-streptavidin (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). The PNA array was incubated at 40°C for 1 h. After hybridization, the PNA array was washed twice for 5 min with PNA array wash buffer. The PNA array was ready for scanning after being dried. Array images were taken using a nonconfocal fluorescent scanner GenePix 4000B (Axon Instruments, Union City, CA) with a typical laser power of 100% and a PMT gain of 700. Scanning was performed at a 635-nm wavelength to detect Cy5 using GenPro Pix 6.0 software (Axon Instruments, Union City, CA). The fluorescence signal intensities indicate the hybridization signals of the probe-target duplexes.

PNA array data analysis. To determine the cutoff values between positive and negative signals, repeated experiments with 28 mutagenic clones were performed. The cutoff value was established as the signal intensity. As a result, the cutoff value was ≥2,000 for the signal intensity and ≥2.5 for the SBR (signal-to-background ratio). When the signal intensity and SBR of wild-type or mutant probes were higher than the cutoff values, the result was considered positive; when the signal intensity and SBR were lower than the cutoff values, the result was considered negative. Thus, if the signal intensity and SBR of wild-type and mutant probes for the same codon was higher than the cutoff value, the result was considered mixed.

Comparative analysis with sequencing assay. To assess the efficiency of this PNA array, we compared the results to those of direct sequencing. The PCR products were purified by using a Labopass PCR purification kit according to the manufacturer's instructions. Purified products were sequenced with selected amplification primers. The results were compared to those of the PNA array. A wild-type, mutated, or mixed status was scored for each of the 11 codons.

RESULTS

Specificity of each target PNA probe. The probes on the PNA array cover the following amino acids: codons L80I/V, I169T, V173L, L180M, A181T/V, T184G/S, S202G/I, M204V/I/S, V207I, N236T, and M250V of HBV reverse transcriptase. Twenty-eight amino acid-specific probes represent wild-type, mutant, or mixed sequence for 11 codons associated with resistance to LMV, ADV, and ETV. The clones obtained by site-directed mutagenesis were used to test the specificity of specific PNA probes for wild-type and mutant sequences at each codon. Mutant clones were correctly identified with specific probes for mutant sequences of each codon. The wild-type clone was correctly identified with wild-type probes for all codons (see Fig. S1 in the supplemental material). The specific PNA probes were specific for wild-type and mutant sequences for each codon associated with resistance to LMV, ADV, and ETV under uniform hybridization conditions.

Detection limit of the PNA array. The detection limit of the PNA array was determined by 10-fold serial dilutions of wild-type and mutant plasmids (10^8 to 10^1 copies of plasmid/ml). The detection limit of the PNA array was 10^2 copies/ml (Fig. 2A). To determine the ability of the PNA array to detect minor populations, we evaluated the detection limit in the mixtures

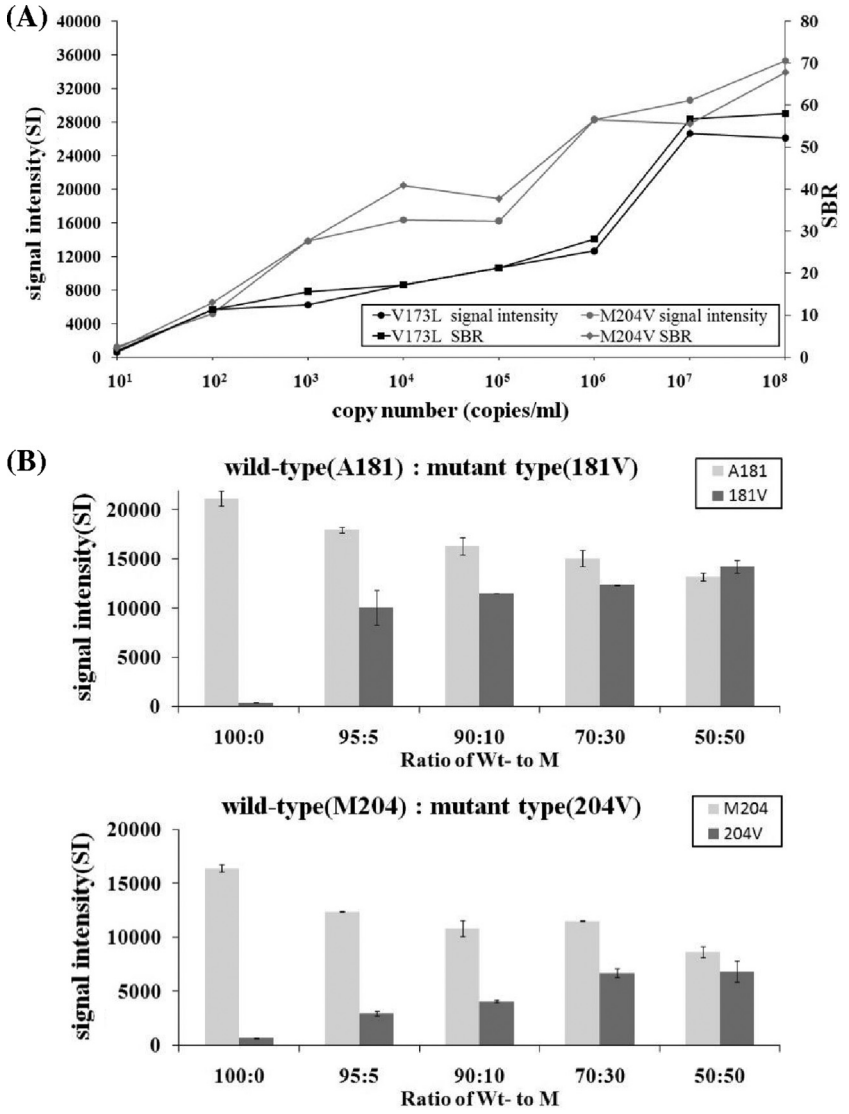

FIG. 2. Detection limit of PNA array for the detection of antiviral-resistant HBV. (A) Serial dilutions were made with 10^8 to 10^1 copies/ml for mutants V173L and M204V. (B) The plasmids of the wild type and mutants A181V and M204V were mixed at 100:0, 95:5, 90:10, 70:30, and 50:50 (wild type to mutant) ratios for concentrations of 10^4 copies/ml.

containing various ratios of wild-type and mutant plasmids. The two plasmids were mixed at 100:0, 95:5, 90:10, 70:30, and 50:50 wild-type-to-mutant ratios for total HBV DNA concentrations of 10^6, 10^5, 10^4, and 10^2 copies/ml (data not shown). At a total HBV DNA concentration of 10^4 copies/ml, the PNA array was able to detect minor population present in 5% of the total viral load (Fig. 2B).

Comparison of PNA array and sequencing. To evaluate the accuracy of the PNA array, the results were compared with direct sequencing for 68 clinical samples (748 amino acid positions). The results obtained for codons 80, 169, 173, 180, 181, 184, 202, 204, 207, 236, and 250 were compared (Table 1).

Data analyses for sequencing versus PNA array. The results were divided into four classes: fully concordant, partially concordant, partially discordant, and completely discordant with sequencing. The results were considered fully concordant if both direct sequencing and the PNA array showed identical wild-type, mutant, or mixture results. The results were considered partially concordant if the PNA array provided additional information not provided by sequencing, meaning that the PNA array showed a mixture while sequencing showed only a wild type or a mutant. The results were considered partially discordant if sequencing provided additional information not provided by the PNA array, meaning that sequencing showed a mixture, while the PNA array showed only a wild type or mutant. The results were considered completely discordant if one test showed a mutant (or wild type) and the other test showed a wild type (or mutant) (8, 19).

(i) Full concordance and partial concordance. Concordance (full and partial) between results of the PNA array and sequencing was observed for 98.3% (735 of 748) of the codons analyzed. Full concordance between results of the PNA array and sequencing was observed for 95.2% of the codons analyzed. Partial concordance between results of the PNA array

TABLE 1. Concordance in amino acid positions between the PNA array for HBV and direct sequencing results

Codon	No. (%) of tests with indicated result							
	Full concordance				Partial concordance (mixed PNA array no. on mixed sequence)	Partial discordance (mixed sequence no. on mixed PNA array)	Complete discordance	Total
	Wild type	Mutant	Mixed	Total				
80	52	7	6	65 (95.6)	2	1	0	68
169	68	0	0	68 (100)	0	0	0	68
173	67	1	0	68 (100)	0	0	0	68
180	52	0	3	55 (80.9)	10	3	0	68
181	61	1	2	64 (94.1)	0	4	0	68
184	67	1	0	68 (100)	0	0	0	68
202	67	0	0	67 (98.5)	0	1	0	68
204	40	11	12	63 (92.6)	3	2	0	68
207	67	0	0	67 (98.5)	0	1	0	68
236	57	0	2	59 (86.8)	8	1	0	68
250	68	0	0	68 (100)	0	0	0	68
Total	666	21	25	712 (95.2)	23 (3.1)	13 (1.7)	0 (0.0)	748 (100)

and sequencing was 3.1% of the codons analyzed. The concordance rate between the PNA array and sequencing was 98.5% at codons 80, 202, 207, and 236, 100% at codons 169, 173, 184, and 250, 95.6% at codon 180, 94.1% at codon 181, and 97.1% at codon 204. For codon 180, the PNA array detected the mixed type but sequencing detected wild-type or mutant sequence only. Because the minor populations were not detected by sequence analysis, the two assays yielded partial concordant results (Table 1).

(ii) Complete discordance and partial discordance. Discordance (complete and partial) between results of the PNA array and sequence analysis was observed for 1.7% (13 of 748) of the codons analyzed. The partial discordance results mean that sequencing showed a mixture of wild-type and mutant sequences, while the PNA array detected a wild-type or mutant probe for each target codon only. Partially discordant results were observed in codons 80, 202, 207, and 236 in one sample each, codon 180 in three samples, codon 181 in four samples, and codon 204 in two samples. Completely discordant results were not observed. Because the minor populations were not detected by the PNA array analysis, the two assays yielded discrepant results (Table 1).

DISCUSSION

The sensitive and early detection of antiviral-resistant mutants is important for monitoring the response to treatment and for effective treatment decisions. In recent years, various methods for the detection of antiviral resistance have been developed and applied (8, 16, 21). Microarray-based techniques have become well established as powerful tools for the diagnosis and monitoring of infectious diseases (5, 13, 22, 28).

In recent years, nucleic acid analogs such as PNAs have been used as probes. PNA arrays and DNA microarrays differ in several aspects. The most important difference is that PNA arrays exhibit superior hybridization characteristics and improved chemical and enzymatic stabilities relative to those of nucleic acids. The PNA:DNA duplex shows a thermal stability that typically is 1.0 to ~1.5°C per base pair higher than that of a DNA:DNA duplex. The changes in melting points of PNA:DNA duplexes are 2.5 to ~8.5°C higher than those of the DNA:DNA duplexes (6, 7, 14). The PNA probes are useful for the detection of mutations or single-nucleotide polymorphisms. PNA is not degraded by nuclease and proteases, because it has a synthetic backbone. Thus, PNA arrays have a shelf-life longer than 12 months when stored in the dark at room temperature (6, 7). Because of the superior properties of PNA, the PNA array gives rise to higher specificity, higher sensitivity, and higher stability than the DNA array in the detection of target sequences. Therefore, the PNA array is important for diagnostic methods (3, 6, 7, 24).

We have developed a PNA array for the detection of point mutations in HBV associated with antiviral resistance that is sensitive, reliable, and simple. Also, we used the single-closed-tube nested PCR method for target amplification in patient samples. The application of nested PCR in medical diagnostics enables high sensitivity for the detection of low target concentrations in clinical samples. This technique can enhance sensitivity but with a high risk of self contamination with amplified DNA when two different tubes are used in the subsequent reactions. To eliminate this risk, single-tube nested PCR can be performed without opening the tube during the entire nested PCR process. The use of nested PCR in a single closed tube diminishes the possibility of contamination and increases the sensitivity and specificity compared to that of a conventional nested PCR (9, 27).

The overall sensitivity of the PNA array assay for the detection of antiviral-resistant HBV was 10^2 copies/ml. The PNA array assay could detect minor populations present at levels as low as 5% of the total viral load.

The performance of the PNA array assay for detecting mutations associated with antiviral resistance was compared to that of direct sequencing for 68 chronically HBV-infected patients who had received antiviral therapy (Table 1). For the 11 amino acids, the concordance rate (full and partial concordance) between both assays was 98.3%. Among them, the partial concordance rate was 3.1%. These results might explain the relatively low sensitivity of the sequencing method. The sequencing method was accurate for less than 20 to ~30% for the detection of low-level mutants (8, 13, 26). Therefore, it is

necessary to analyzed multiple clones by cloning for detecting minor populations and determining the heterogeneity. In contrast, for 1.7% of the codons, the PNA array and sequencing were observed to have partially discordant results. There are a total of 13 partially discordant results, indicating that sequencing detects mixtures while PNA detects only the wild type. Eleven of 13 results that showed partial discordance between the tests were observed for seven codons each. Eleven partially discordant results were present in three different specimens. Two results showed partial discordance at codons 181 and 207. Eleven results for seven codons for three samples yielded unambiguous sequencing results. One yielded unambiguous sequencing results at codons 80, 180, 181, and 236. Another yielded unambiguous sequencing results at codons 180, 181, and 204. The other yielded unambiguous sequencing results at codons 180, 181, 202, and 204. The sequencing results were unambiguous for more than one codon, and a repeat test procedure starting from the sample extraction step was performed. We performed resequencing analysis; thus, we performed sequencing analysis a total of three times. Eleven of the discrepant results showed low reproducibility for the three sequencing analyses. Sequencing results were observed for 11 mixtures in the first analysis, whereas mutant sequence was not observed with the second and third sequencing series. For two samples, mutant probe results for codons 181 and 207 with the PNA array were not observed. In one case, sequence analysis revealed other polymorphisms at codon 181 (TCT) and codon 207 (CTG) that interfered with the annealing of the PNA probes for the detection of mutants. Thus, the PNA array result for codons 181 and 207 showed a wild-type spot only and a positive signal for the wild-type probe only. A discordant result was not observed. These results indicate that the PNA array assay is more sensitive and more reliable. Moreover, using the PNA array was a very simple process of DNA extraction, one-step PCR, hybridization, and scanning.

In conclusion, we established a PNA array assay for the detection of a point mutation in HBV associated with antiviral resistance. The specificity and sensitivity of the PNA probes has been demonstrated, especially with regard to the detection limit for the minor population in a mixture. The PNA array is a rapid, sensitive, and easily applicable assay for the detection and monitoring of antiviral-resistant mutations in HBV. The PNA array will be a useful and powerful diagnostic tool for the detection of point mutations or polymorphisms.

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