Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII

RIMA YOUIL[†], BORRIES W. KEMPER[‡], AND RICHARD G. H. COTTON[†]

†Olive Miller Laboratory, Murdoch Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052, Australia; and ‡Institute for Genetics of the University of Cologne, Weyertal 121, 50931 Cologne 41, Germany

ABSTRACT Each of four possible sets of mismatches (G·A/C·T, C·C/G·G, A·A/T·T, and C·A/G·T) containing the 8 possible single-base-pair mismatches derived from isolated mutations were examined to test the ability of T4 endonuclease VII to consistently detect mismatches in heteroduplexes. At least two examples of each set of mismatches were studied for cleavage in the complementary pairs of heteroduplexes formed between normal and mutant DNA. Four deletion mutations were also included in this study. The various PCR-derived products used in the formation of heteroduplexes ranged from 133 to 1502 bp. At least one example of each set showed cleavage of at least one strand containing a mismatch. Cleavage of at least one strand of the pairs of heteroduplexes occurred in 17 of the 18 known single-base-pair mutations tested, with an A·A/T·T set not being cleaved in any mismatched strand. We propose that this method may be effective in detecting and positioning almost all mutational changes when DNA is screened for mutations.

The detection of mutations is important, particularly in the diagnosis of inherited diseases. Changes in the DNA sequences of a gene can be harmful and it is important in our understanding of human genetics that we are able to identify and classify these alterations and the phenotypic changes that they induce. Consequently, the need for a reliable method for the detection of mutations in DNA to avoid repetitive sequencing of kilobase lengths of DNA has led to the development of a number of different screening methods that have both positive and negative attributes (see ref. 1 for a review of current mutation detection methods). Thus, the search for a reliable and efficient approach to the detection of known and unknown mutations continues.

The resolvases are an important group of enzymes that are responsible for catalyzing the resolution of branched DNA intermediates that form during genetic recombination. Their mode of action is directed by bends, kinks, or DNA deviations. These enzymes have their effect close to the actual site of DNA distortion (2). T4 endonuclease VII, the product of gene *49* of the bacteriophage T4 (2), is a resolvase that has been well characterized (3–5). It was the first enzyme shown to resolve Holliday structures (2). It has also been shown to recognize cruciforms (2, 3) and loops (6). It may also be involved in very short patch repair (5). Its cleavage characteristics involve it cleaving 3' and within 6 nt from the point of DNA perturbation—causing double-stranded breakage (2, 5). T4 endonuclease VII has been shown to cleave single-base-pair mismatches in model experiments with synthetic oligonucleotides up to ≈43 bp (7). This work examines the ability of the enzyme to detect mutations rather than its ability to cleave specific mismatches. Thus, when mutant and wild-type homoduplexes that differ by a single base pair are melted and hybridized, two heteroduplex species are formed containing two pairs of mismatched bases. The mutation would be detected if any one strand containing one of the four mismatched bases were cleaved. There are four classes of pairs of mismatched bases (type 1, G·A/T·C; type 2, G·T/A·C; type 3, C·C/G·G; type 4, T·T/A·A) and at least two members of each were tested. Only one example showed no cleavage in any of the strands.

MATERIALS AND METHODS

Enzyme and Buffers. T4 endonuclease VII was prepared from an overexpressing *Escherichia coli* K38 transformant containing gene *49* of T4 phage as described by Kosak and Kemper (7). Stock solutions were at 3700 units/μl as determined by Kosak and Kemper (7), where 1 unit is defined as that amount of enzyme that catalyzes degradation of 50% of very fast sedimenting DNA. The reaction buffer used in the assay was prepared as a 10× concentrate (7). The enzyme dilution buffer was prepared as described (7). Assay conditions required between 250 and 3000 units of T4 endonuclease VII depending on the specific DNA being tested. The annealing buffer was prepared as a 2× concentrate (1.2 M NaCl/12 mM Tris·HCl, pH 7.5/14 mM MgCl₂) as described (8). The kinase buffer, 1× TE (pH 8.0), and the formamide/urea loading dye were prepared as described (9).

DNA Preparation. The DNA used in these experiments was amplified by PCR from genomic DNA [β-globin, phenylalanine hydroxylase (PAH), α₁-antitrypsin], plasmid DNA (21-hydroxylase and the mouse mottled Menkes gene), or cDNA [pyruvate dehydrogenase E1α subunit (PDH E1α), dihydropteridine reductase, and the rhodopsin gene]. Each region contained an example of a known mutation except for the mouse mottled Menkes gene and dihydropteridine reductase mutations, which were previously unpublished.

Each DNA sample was prepared by PCR amplification. The β-globin gene (M8, mutation at nt 26 exon 2; M14, mutation at nt −87; M16, sickle mutation; M21, mutation at nt 17 exon 1) was amplified by using primers a and b (10). A larger segment of the β-globin gene (M15), which contains mutations at nt 745, 16, 74, 81, and 666 within IVSII, was amplified by using primers c and d as described (10). The α₁-antitrypsin gene (M4, mutation at nt 9989) was amplified as described (11). The PDH E1α gene (M1, F205L; M19, K387fs; M20, S312fs) was amplified by using primers PDH-P and PDH-E as described (12). The PAH gene M5 (homozygous mutation at IVS12 nt 1), M6 (heterozygous mutation at IVS12 nt 1), and M7 (R408W) was PCR amplified by using primers A and B as described (13). The PAH gene (M13, F39L exon 2) was PCR amplified by using the primers 5'-d(GCA TCT TAT CCT GTA GGA AA)-3' and 5'-d(AGT ACT GAC CTC AAA TAA GC)-3'. The PCR conditions were 105 s at 95°C, 150 s at 58°C, and 3 min at 72°C for 35 cycles. The 340-bp section of the

Abbreviations: PAH, phenylalanine hydroxylase; PDH, pyruvate dehydrogenase; EMC, enzyme mismatch cleavage; CCM, chemical cleavage of mismatch.

Table 1. Summary of mutations tested for by EMC

Type	Sample	Base change	Sequence context	Mismatch set	Mismatch detected	Total fragment length, bp	Nonspecific cleavage†	Fig.
1	M1 (Hom)	C → A	ATTCGAA	A·G/T·C	C·T	797	−	1
1	M2‡ (Hom)	A → C	CCCAATC	A·G/T·C	A·G/T·C	340	−	
2		T → C§	CACTTGC	G·T/C·A	ND¶		−	
2	M3 (Hom)	T → C§	CACTTGC	G·T/C·A	G·T	178	−	
2	M4 (Hom)	G → A	GACGAGA	G·T/C·A	C·A	220	+	
2	M5 (Hom)	G → A§	ACAGTAA	G·T/C·A	G·T/C·A	245	+	2
2	M6 (Het)	G → A§	ACAGTAA	G·T/C·A	G·T/C·A	245	+	
2	M7 (Het)	C → T	TACCTCG	G·T/C·A	G·T/C·A	245	+	
2	M8 (Hom)	C → T	ACCCAGA	G·T/C·A	G·T	627	−	
2	M9 (Het)	A → G	CCAATGC	G·T/C·A	?	1300	−	
2	M10‖ (Het)	T → C	AGCTCTT	G·T/C·A	G·T	779	−	
2	M11‖ (Hom)	A → G	TGGAGGA	G·T/C·A	?	1502	−	
2	M12‖ (Hom)	C → T	GATCATT	G·T/C·A	?	1502	−	
3	M13 (Het)	C → G	CTTCTCA	C·C/G·G	C·C**	133	+	3
3	M14 (Hom)	C → G	CACCCTA	C·C/G·G	C·C	627	−	
3	M15‡ (Het)	C → G	CAGCTAC	C·C/G·G	C·C/G·G	1377	−	
3		C → G	GACCCTT	C·C/G·G	C·C	1377	−	
1		G → T	GGAGAAG	A·G/T·C	A·G/T·C	1377	−	
2		C → T	TAACAGG	G·T/C·A	G·T/C·A	1377	−	
2		T → C	TATTTCT	G·T/C·A	G·T/C·A	1377	−	
4	M16 (Het)	A → T	CTGAGGA	A·A/T·T	ND	627	−	
4	M17 (Hom)	T → A	TCATCTG	A·A/T·T	A·A/T·T	204	−	4
Del	M18‖ (Hom)	Del 33 bp	TAG-AGG	33-bp loops	?	1502	−	
Del	M19 (Hom)	Del 2 bp	TTT-GTC	AA/TT loops	TT loop**	797	−	
Del	M20 (Hom)	Del 7 bp	GGA-AGT	7-bp loops	?	797	−	
Del	M21 (Het)	Del 1 bp	CTG-GGA	A/T loops	ND	627	−	

Source of mutation was either genomic, plasmid, or cDNA. Samples were either homozygous (Hom) or heterozygous (Het). Details of genes involved are given in the text. Sequence context of the sense strand of normal DNA is shown. Underlined base denotes nucleotide involved in the base change. ND, no cleavage was seen in any one of the four strands containing a mismatched base; ?, cleavage was observed at the mismatches but the strand cleaved could not be determined; Del, deletion (bases given flank the mutation).
†Substantial cleavage was seen in homoduplex control DNA (as well as heteroduplex) but the exact position has not been determined.
‡More than 1 base change was present in the fragment studied.
§Exactly the same mutation has been tested for the respective genes.
¶This mutation at nt 118 was not detected when in the presence of a second mutation at nt 138. However, it was detected in a shorter fragment that did not include the mutation at nt 138 (M3).
‖These mutations were previously unknown.
**Postdigestion end-labeling was performed on these fragments also.

21-hydroxylase B gene (M2, mutations at nt 118 and 138) was amplified by using the primers 5'-d(CTG CTG TGG AAC TGG TGG AA)-3' and 5'-d(ACA GGT AAG TGG CTC AGG TC)-3'. The 178-bp section of the 21-hydroxylase B gene (M3, mutations at nt 118) was amplified by using the primers 5'-d(GCT CTT GAG CTA TAA GTG G)-3' and 5'-d(GGG AGG TCG GGC TGC AGC A)-3'. The 21-hydroxylase A gene (M17, mutation at nt 1004) was amplified by using the primers 5'-d(CTG CAC AGC GGC CTG CTG AA)-3' and 5'-d(CAG TTC AGG ACA AGG AGA GG)-3'. The PCR conditions for the 21-hydroxylase A and B genes were 105 s at 95°C, 150 s at 62°C, and 3 min at 72°C. The dihydropteridine reductase gene (M10, L74P at nt 245) was amplified by using the primers GD and F as described (14). The rhodopsin gene (M9, mutation at codon 15, nt 44) was amplified as described (15). The mouse mottled Menkes gene (M11, mutation at nt 3662; M12, mutation at nt 3367; M18, mutation at nt 4516) was amplified as described (16).

The PCR products were electroeluted onto Whatman I paper in a 1.5% agarose gel and then eluted with 1× TE (pH 8.0). In all cases, the corresponding normal DNA was end-labeled with [γ-³²P]ATP using 5' T4 polynucleotide kinase (Boehringer Mannheim). After the kinase treatment, the DNA was ethanol precipitated and the pellet was washed three times with 70% ethanol to wash away the major portion of unincorporated label. The pellet was resuspended in distilled water to give ≈5 ng of end-labeled DNA per μl.

Heteroduplex formation was performed in 50 μl (total volume) containing 1× annealing buffer as described (8) except that the annealing temperature was at 65°C for 1 hr followed by 20 min at room temperature. Calculations of DNA concentration were based on 50–60 ng of unlabeled DNA (10× excess) and 5 ng of end-labeled DNA per single reaction. Heteroduplexes were prepared in bulk in a 50-μl volume and the pellet was resuspended in the appropriate volume of distilled water. For example, if six reactions were required then 300 ng of mutant DNA and 30 ng of labeled wild-type DNA was used. After the heteroduplex reaction, the pellet was resuspended in 30 μl of distilled water (i.e., 5 μl of distilled water is taken per single reaction). An identical procedure was performed in order to prepare labeled homoduplex DNA for the control studies except that an excess amount of unlabeled wild-type DNA was hybridized with the labeled wild-type DNA. This strategy allows two of the four mismatched bases (those present in the labeled strand) to be tested for cleavage by the enzyme.

Enzyme Mismatch Cleavage (EMC). Five microliters of the labeled homoduplex or heteroduplex DNA (50–60 ng) was added to 39 μl of distilled water and 5 μl of 10× reaction buffer, all kept on ice. The reaction was initiated by the addition of 1 μl of the enzyme (100–3000 units/μl as specified). The stock solution of enzyme was diluted to the required activity in the enzyme dilution buffer. After addition of the enzyme, the tubes were spun briefly and incubated at 37°C for 1 hr unless otherwise specified. In the case of controls, the enzyme was replaced with 1 μl of the enzyme dilution buffer and these were incubated. After incubation, the samples were ethanol precipitated, washed in 70% ethanol, dried briefly, and

thoroughly resuspended by vortex mixing in 5 μl of form-amide/urea loading dye. The 5-μl samples were heated to 100°C and immediately loaded onto an 8% urea/acrylamide sequencing gel. Cleavage products were visualized by autora-diography, and the sizes of the products were compared with radiolabeled ϕX174 *Hae* III size marker.

Postdigestion End-Labeling. Heteroduplexes were prepared in a 1:1 ratio of unlabeled normal and unlabeled mutant DNA. Heteroduplex formation was identical to that described above except that 25 ng of wild-type DNA and 25 ng of mutant DNA were mixed together and annealed to give 50 ng of duplex DNA per reaction mixture. Fifty nanograms of wild-type DNA was used for the homoduplex control. Enzyme digestion was performed on 50 ng of unlabeled duplex DNA and the products of digestion were end-labeled in a total of 10 μl of 1× kinase buffer, 2 units of 5' polynucleotide kinase, and 1 μl of a 1:10 dilution of fresh [γ-^{32}P]ATP. After incubation (45 min at 37°C), the enzyme was denatured at 70°C for 10 min and the reaction mixture was ethanol precipitated. The pellet was washed three times in 70% ethanol, dried briefly, and resus-pended in 5 μl of formamide loading dye.

Chemical Cleavage of Mismatch (CCM). CCM was used as a control method to ensure heteroduplexes had formed and was performed as described (8).

RESULTS

The mutations were chosen to cover at least two examples of each of the four types of single-base-pair mismatch combina-tions possible when heteroduplexes are formed between mu-tant and wild-type genes differing by any of the 12 possible single-base changes. Heteroduplex loops resulting from dele-tions in the mutant DNA were also examined. Results of only one example of each type are given. Results of the other examples are summarized in Table 1. Mutations studied were isolated such that only one mutation occurred in the length studied unless otherwise stated.

Type 1 (Mismatch Set G·A/T·C). M1 contains a homozy-gous C → A mutation 87 bp away from the 5' end of the section of the gene studied (F205L). Using end-labeled wild-type probe, the resulting heteroduplexes contain C*·T and A·G* mismatches. The asterisk designates that the base is present in the labeled strand. After CCM, only the 87-bp fragment was observed on denaturing acrylamide gel since hydroxylamine modifies only the mismatched C. With the EMC, only a single band slightly larger than the 87-bp fragment was observed, suggesting cleavage near the C* in the C*·T mismatch (Fig. 1).

Type 2 (Mismatch Set G·T/A·C). A 245-bp section of the PAH gene (M5) was amplified from genomic DNA of a patient homozygous for a G → A mutation (IVS12 nt 1). This mutation occurred 191 bp from the 5' end of the PCR product. End-labeled normal DNA was hybridized with unlabeled mutant DNA. After enzyme cleavage, two bands, one slightly larger than the 191-bp band and another slightly larger than the 54-bp band, were observed resulting from cleavage near the G* in the G*·T mismatch and near the C* in the A·C* mismatch, respectively (Fig. 2).

Type 3 (Mismatch Set C·C/G·G). The PAH mutation (M13) used here involves a heterozygous C → G mutation at base 57 in exon 2, 73 bp from the 5' end of the 133-bp section of the gene studied. Heteroduplex formation between labeled nor-mal and unlabeled mutant DNA produced C*·C and G·G* mismatches as well as homoduplexes corresponding to the normal and mutant DNA. CCM using hydroxylamine allowed detection of only the C*·C-containing heteroduplex. Only the 73-bp 5'-end-labeled sense strand derived from the wild-type probe was observed on acrylamide gel electrophoresis. EMC of the same heteroduplex showed a similar pattern except that a band slightly larger than the expected 73-bp fragment was observed (Fig. 3A). This band results from cleavage near the

C*·C heteroduplex. It is clear that the enzyme does not seem to recognize the mismatched G* in the labeled wild-type strand since a 60-bp fragment was not observed. EMC in this section of the PAH gene results in the production of a fragment due to nonspecific cleavage.

Postdigestion end-labeling of this same DNA showed bands slightly larger than the expected 60- and 73-bp bands on the autoradiograph (Fig. 3B), suggesting that there was double-stranded cleavage of the C·C-containing heteroduplex. Cleav-age by T4 endonuclease VII results in 3' OH and 5' PO$_4$ ends. Only the 5' OH ends (primer ends) will label with [γ-^{32}P]ATP using 5' T4 polynucleotide kinase. Hence, the fragments 3' to the cleavage will NOT be observed on autoradiography. Note that the same nonspecific fragment observed in Fig. 3A is present here also.

Type 4 (Mismatch Set A·A/T·T). A 204-bp section of the 21-hydroxylase A gene (M17) was PCR amplified from plas-
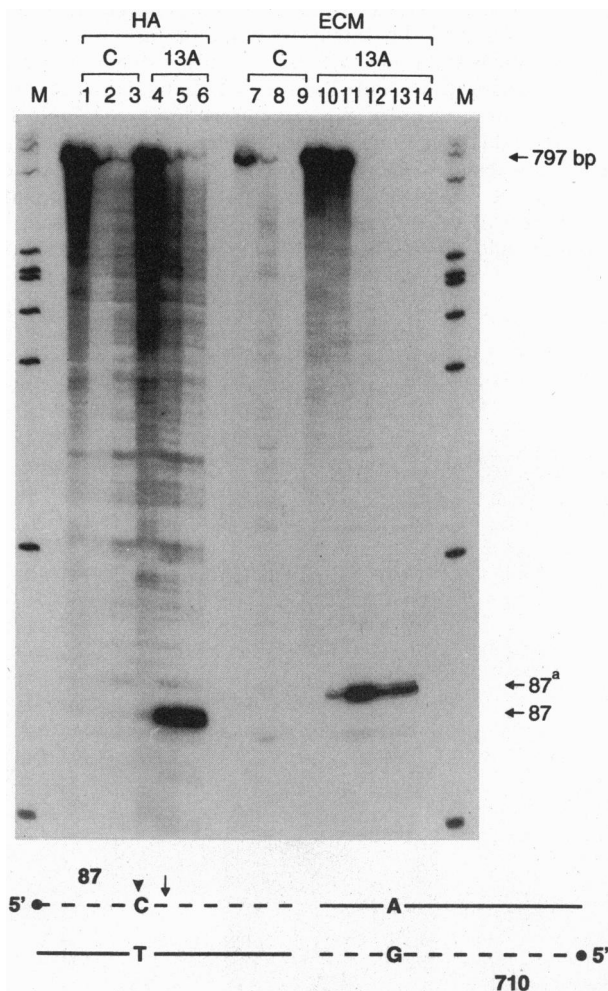

FIG. 1. Autoradiograph of CCM and EMC analysis of the PDH E1α gene (M1) containing a homozygous C → A mutation. Lanes: 1–3, samples of control homoduplex DNA (C) after incubation with hydroxylamine (HA) for 0, 1, and 2 hr; 4–6, samples of test hetero-duplex DNA (13A) after incubation with hydroxylamine for 0, 1, and 2 hr; 7–9, samples of C after incubation with 0, 1000, and 3000 units of T4 endonuclease VII; 10–14, samples of 13A after incubation with 0, 250, 1000, 2000, and 3000 units of T4 endonuclease VII. Scheme below represents two types of heteroduplexes that are formed (as described in the text). Broken line, wild-type DNA strands; straight lines, mutant DNA strands; arrows, actual enzyme cleavage sites on the end-labeled strand in each heteroduplex; ●, label; arrowhead, actual cleavage site of the CCM reaction. Superscript a refers to band sizes observed by EMC being slightly larger than the expected band sizes as determined by CCM. Lanes M, end-labeled marker ϕX174 *Hae* III.

mid DNA. This mutant DNA contained a homozygous T → A mutation at base 1004 of the gene, 110 bp from the 5' end of the section of the gene studied. This results in a heteroduplex species containing both A·A* and T*·T mismatches. EMC on this sample with end-labeled probe showed two bands: one slightly greater than 110 bp and the other slightly greater than 94 bp (Fig. 4). This confirms that the enzyme recognized both strands of the probe. The intensity of the products on autoradiography shows that the enzyme recognizes the A·A* mismatch with greater efficiency than the T*·T mismatch. CCM on this sample showed only the 110-bp fragment obtained after modification and cleavage of the mismatched T base in the sense strand of the probe (Fig. 4).

DISCUSSION

A total of 3 type 1, 13 type 2, 4 type 3, 2 type 4, and 4 deletion mutations have been tested by the enzyme cleavage method in this study. Four of the single-base-pair mutations detected were previously unknown. Of the 18 known single-base mutational changes tested, only 1 (an A → T change) did not show cleavage of any strand of the two heteroduplexes (Table 1). This may be due to a sequence context feature since of the four single loops tested (generated by deletions) only one did not show cleavage of any strand, and that one involved the same base as the single-base mutational change A → T present in M16. We would like to investigate the reasons for nondetection
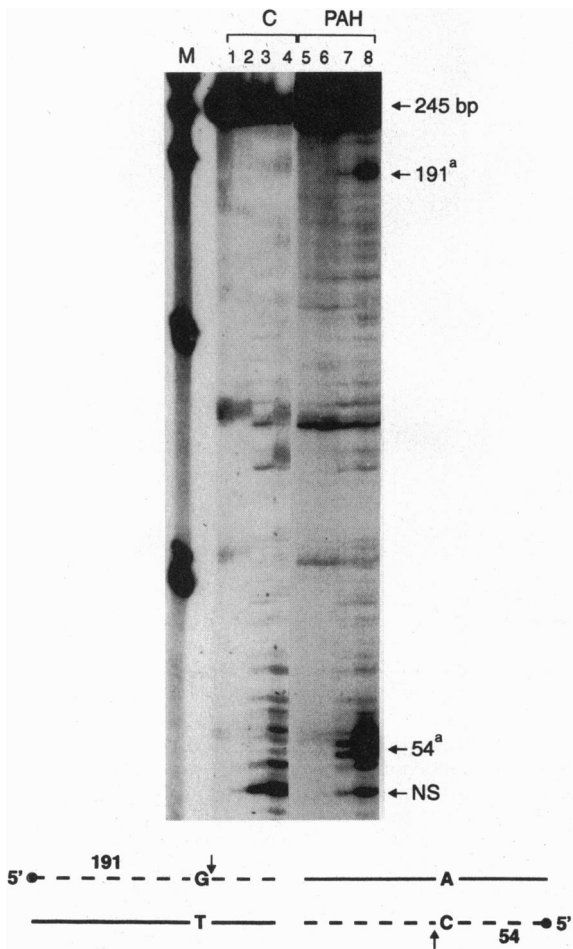

Fig. 2.   Autoradiograph of EMC analysis of the PAH gene (M5) containing a homozygous G → A mutation. Lanes: 1–4, samples of control homoduplex DNA (C) after incubation with 0, 250, 500, and 1000 units of T4 endonuclease VII; 5–8, samples of test heteroduplex DNA (PAH) after incubation with 0, 250, 500, and 1000 units of T4 endonuclease VII. Scheme below is as in Fig. 1.
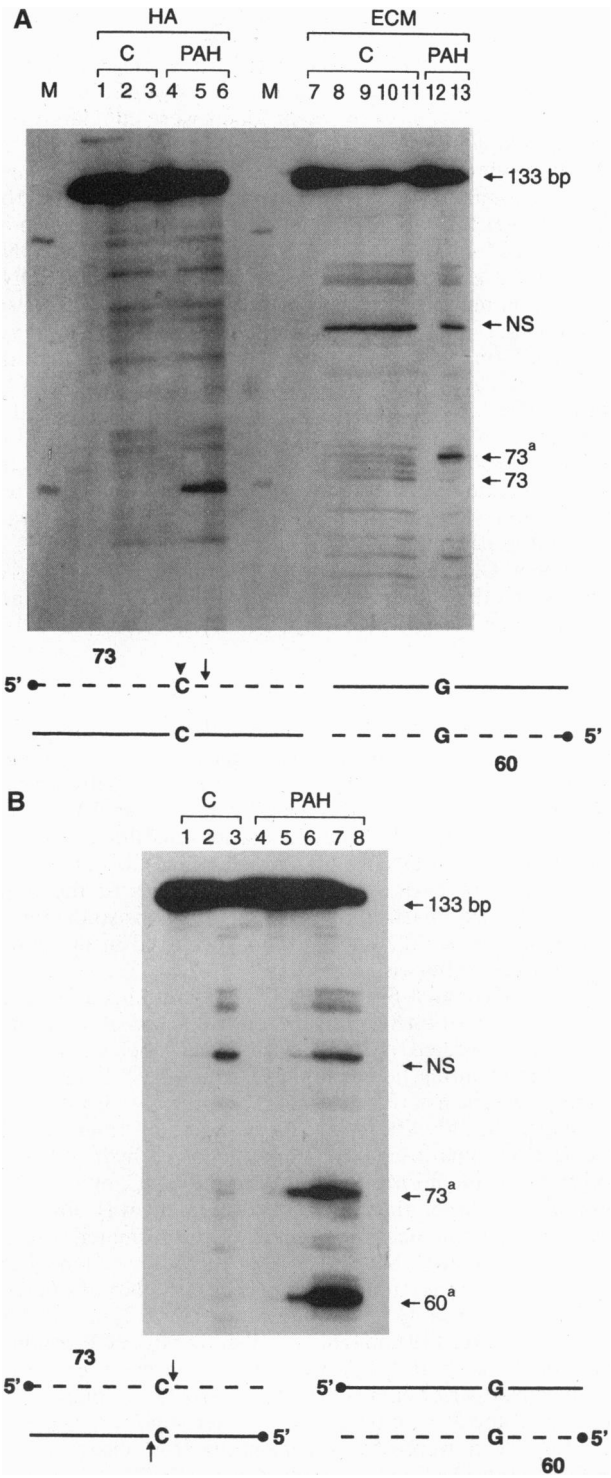

Fig. 3.   (*A*) Autoradiograph of CCM and EMC analysis of the PAH gene (M13) containing a heterozygous C → G mutation in exon 2. Lanes: 1–3, samples of control homoduplex DNA (C) after incubation with hydroxylamine for 0, 1, and 1.5 hr; 4–6, samples of test heteroduplex DNA (PAH) after incubation with hydroxylamine for 0, 1, and 1.5 hr; 7–11, samples of C after incubation with 0, 1000, 2000, 2500, and 3000 units of T4 endonuclease VII; 12 and 13, samples of PAH after incubation with 0 and 1000 units of T4 endonuclease VII. (*B*) Autoradiograph of postdigestion end-labeling (as described in the text) of the PAH gene (M13) containing a heterozygous C → G mutation in exon 2. Lanes: 1–3, samples of control homoduplex DNA (C) after incubation with 0, 250, and 1000 units of T4 endonuclease VII; 4–8, samples of test heteroduplex DNA (PAH) after incubation with 0, 100, 250, 500, and 1000 units of T4 endonuclease VII. Schemes below are as in Fig. 1.

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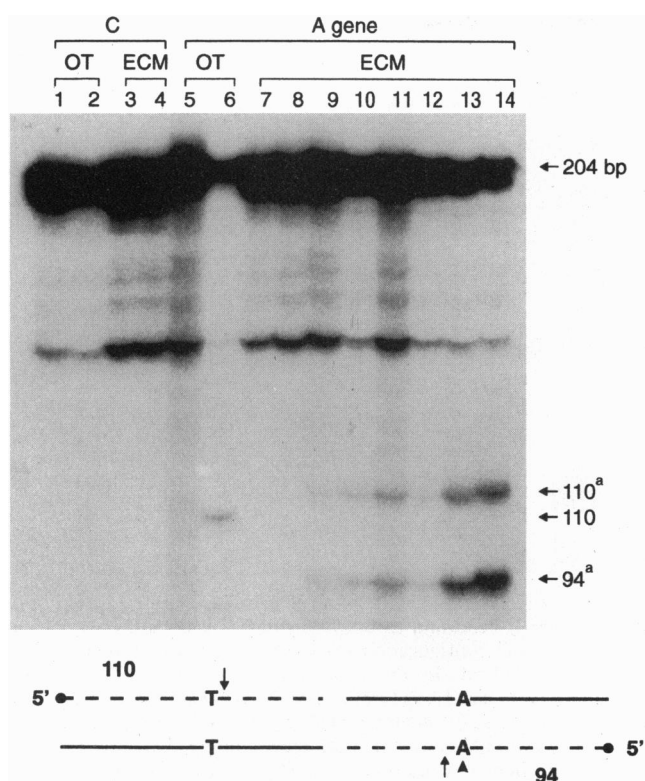*Proc. Natl. Acad. Sci. USA* 92 (1995)   91

FIG. 4. Autoradiograph of CCM and EMC analysis of the 21-hydroxylase A gene (M17) in a plasmid containing a homozygous T → A mutation. Lanes: 1 and 2, samples of control homoduplex DNA (C) after incubation with osmium tetroxide (OT) for 0 and 5 min; 3 and 4, samples of C after incubation with 0 and 250 units of T4 endonuclease VII; 5 and 6, samples of test heteroduplex DNA (A gene) after incubation with OT for 0 and 5 min; 7 and 8, samples of A gene after incubation with 0 and 250 units of T4 endonuclease VII; 9–11, samples of A gene after incubation with 500 units of T4 endonuclease VII for 1, 3, and 16 hr; 12–14, samples of A gene after incubation with 1000 units of T4 endonuclease VII for 1, 3, and 16 hr at 37°C. Scheme below is as in Fig. 1.

of any mutations and attempt to change conditions appropriately to allow detection. Similarly, we would like to investigate the reasons for nonspecific cleavage present in some cases (see Table 1 and Fig. 3).

Our results show that in about half the cases studied, detection was observed by the cleavage of one of the heteroduplexes in the set. For example, the heteroduplex containing the A·G mismatch was cleaved in M2, but we relied on cleavage of the heteroduplex containing the reciprocal T·C mismatch for detection in M1 (Table 1). This gives further support to the view that T4 endonuclease VII is dependent on sequence context as well as DNA structure (5, 17). It is also clear from Table 1 that the mismatch pair generally considered to be the most thermostable (G·T) is recognized efficiently by T4 endonuclease VII where 8 of 13 type 2 mismatches tested showed cleavage of G·T-containing heteroduplex. In these cases, detection depended either solely (in three cases) or in conjunction with recognition of the complementary C·A mis-

match pair (in five cases). At the other end of the scale, the mismatch pair considered to be one of the least thermostable (C·C) was recognized by T4 endonuclease VII in all four cases tested in this study.

The postdigestion end-labeling method described here was developed to apply these findings to screen lengths of DNA in the most effective manner. Most experiments were performed with excess unlabeled target DNA over labeled probe DNA to form duplexes before cleavage. For simple and practical use, we propose forming duplexes between equimolar mutant and wild-type DNA, cleaving and then kinase labeling all 5′ OH ends before electrophoresis. This allows assay of each strand for cleavage without probe production, thus maximizing the chances of detecting mutations. When using this method, two bands were always observed resulting from the labeling of all the free 5′ OH ends of the cleavage products.

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