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Detection of single base mismatches of thymine and cytosine residues by potassium permanganate and hydroxylamine in the presence of tetralkylammonium salts

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ABSTRACT

In the presence of tetramethylammonium chloride, potassium permanganate specifically modifies mismatched thymines. Similarly, the modification of mismatched cytosines by hydroxylamine was enhanced by tetraethylammonium chloride. Modification followed by piperidine cleavage permits specific identification of the T and C mismatches and by extension, when the opposite DNA strand is analyzed, of A and G mismatches as well. These reactions can be performed conveniently with DNA immobilized on Hybond M-G paper. We describe conditions that exploit these reactions to detect mismatches, e.g. point mutations or genetic polymorphisms, using either synthetic oligonucleotide probes or PCR amplification of specific genomic DNA sequences.

INTRODUCTION

The convenient detection of point mutations or polymorphisms is important in many fields of research including medical and evolutionary genetics, as well as for diagnostic purposes. Three major procedures are currently in use for detecting single base pair differences in the form of mismatches: a. Denaturing gradient gel electrophoresis, exploiting the differential mobility of native and partially denatured DNA.DNA heteroduplexes (1), b. RNase A cleavage of RNA.RNA or RNA.DNA heteroduplexes (2.3), and c. Chemical reactions of DNA with osmium tetroxide or hydroxylamine, which lead to preferential cleavage at T or C mismatches, respectively, upon subsequent treatment with piperidine (4). A recent comparison of the three methods (5) indicates that denaturing gel electrophoresis is the most reliable and informative procedure, although it is somewhat more cumbersome. Chemical reactions are convenient, but a disadvantage of osmium tetroxide is its hazardous nature. Because of the convenience of chemical reactions, we have sought an alternative to osmium tetroxide for detection of T mismatches.

The interactions of tetralkylammonium salts with nucleic acids have been studied for a number of years. Their cations have several interesting chemical properties: preferential binding to AT base pairs, hydrophobic side groups and high molecular weight resulting in low screening of the phosphate charges in DNA (6). These properties have been exploited in hybridization reactions, to abolish the preferential melting of AT versus GC base pairs (7), greatly facilitating hybridization studies with oligonucleotide probes (8,9). We have found that tetramethylammonium chloride (Me_4NCl) accentuates the reactivity of T mismatches (and to a lesser degree C mismatches) towards potassium permanganate, a chemical much less hazardous and unstable than osmium tetroxide. Similarly, tetraethylammonium chloride (Et_4NCl) can accentuate the reactivity of C mismatches towards hydroxylamine. We have also demonstrated that these reactions can be made compatible with the recently introduced Hybond M-G (Amersham) chemically modified paper(10). Thus, we propose tetralkylammonium-enhanced chemical modification on solid support as a very convenient method for detection of point mutations and polymorphisms.

MATERIALS AND METHODS

Oligonucleotides and sequencing

Oligonucleotides were synthesized in an Applied Biosystems 380B synthesizer and eluted from the controlled pore glass columns with ammonium hydroxide (12h heat elution at 50°C). The deprotected sample was lyophilized, resuspended in 0.2 ml water and purified in a 20% acrylamide/7M urea gel. Three pairs of complementary strands representing wild type and mutated versions of the *Drosophila melanogaster s15* chorion gene promoter were synthesized. In all cases, one oligonucleotide was 34 bp long and had the sense sequence (−113) GCAATTATG-AAATGCNATTTCTGGGCTGAAACAG (−80); the numbers

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indicate location relative to the cap site of the gene(11), and the variable nucleotide (N, at −98) was C (34C oligonucleotide, wild type), or T (34T), or A (34A). The other oligonucleotides were 31 bp long and had the antisense sequence (−81) TGTTTCA-GCCCAGAAATNGCATTTCATAATT (−118), with the variable nucleotide (N, at −98) being G (31G,wild type), or A(31A), or T(31T). In addition, a 63-mer oligonucleotide of the sequence TCAAATGTTGAGTATATTCCAGCCGGGC-AATTATCAAATGCCTTTTCTGGGCTAAAACTGACC was synthesized representing a mutated version of the same promoter (3 base substitutions indicated by underlining). A 76 bp heteroduplex was prepared by appropriately annealing the amplification products of two homologous regions of the *s15* gene promoter from *Drosophila grimshawi* and *Drosophila silvestris*. Oligonucleotides were sequenced with a set of chemical reactions we and others (12) have optimized for higher yield and specificity in cleavage by adjusting mainly the temperature, time and concentration of modifying chemicals:

for **A**: 20mM acetic acid/6mM potassium tetrachloropalladate (II) at 37°C for 1.5h, or 1.6M formaldehyde /4M ethanolamine at 37°C for 1h .

for **G** : DMS (1μl in 200 μl cacodylate buffer) at 37°C for 6 min.

for **A+G**: 20mM acetic acid .

for **C**: 3M hydroxylyamine at 37°C, or 4M at room temperature, for 20 min.

for **T**: 0.2mM potassium permanganate ($KMnO_4$) /10mMTris-HCl pH8.0 at 37°C for 15 min.

Oligonucleotide primers for the Polymerase Chain Reaction, PCR(13), were similarly synthesized but were used unpurified.

Oligonucleotide duplexes

Homoduplexed or heteroduplexed oligonucleotides were formed by annealing the appropriate strands. In all experiments, the 5′ end labelled strand is listed first: e.g. 34C/31T indicates that the labelled probe is the 34 C oligonucleotide, and the unlabelled strand (which may be in solution or immobilized on paper) is 31T. Labelling was performed with ^{32}P using T4 polynucleotide kinase and [γ^{32}P]dATP (6000Ci/mmole) in 20μl total volume, followed by kinase inactivation (at 65°C for 10 min). Unincorporated label was removed by passing through a NENsorb (DuPont) mini-column. Lower strand was added together with NaCl (150mM final) and the mixture was left to anneal (e.g overnight in a 65°C oven which was turned off). Double stranded oligonucleotides were separated from remaining single strand by nondenaturing polyacrylamide gel electrophoresis. DNA bands were visualized by autoradiography, isolated and extracted at room temperature for 1h in 0.3M sodium acetate /Tris-EDTA buffer and precipitated with ethanol.

For the construction of the multiply mismatched duplex the end labelled 63-mer was mixed with 10-fold molar excess of a 150 bp unlabelled restriction fragment from the wild type *s15* promoter including the oligonucleotide sequence, heated to 95°C for 5 min and annealed for 1.5 h at 37°C in 250mM NaCl/ 5mM $MgCl_2$/ 5mM Tris-HCl pH8.0.

Duplexes using PCR products

Genomic DNA (0.1 μg) from two individuals was subjected to amplification with 2.5 units of *Taq* polymerase in a reaction mixture (100 μl total volume) including 100pmoles of each primer, 200μM in each dNTP, 50mM KCl, 20mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$ and 0.01% gelatin (Gene-Amp Kit, Perkin Elmer Cetus). Whenever labelled probes were synthesized, labelled and unlabelled primer were mixed in a ratio of 1:1 (50pmole each). The mixture was subjected to an initial template denaturation step at 94°C for 1 min 30 sec (in a Perkin Elmer Cetus Thermocycler) followed by 35 cycles of the profile: 2min at 65°C(annealing), 2min at 72°C (extension), 1min at 94°C(denaturation). The final product was analyzed in a 3% NuSieve/1% ME agarose 1× TBE gel and the DNA of interest was cut and isolated by electroelution, passage through a NACS PREPAC (BRL) mini-column and ethanol precipitation. For each individual we performed a reaction for each set of primers. In every case the primer that amplified the strand containing the T polymorphism was ^{32}P end labelled with [γ-^{32}P]dATP and T4 polynucleotide kinase, cleaned by passing through a NENsorb (DuPont) mini-column and lyophilized. Labelled products were mixed with 5−6 fold molar excess of appropriate unlabelled ones. The mixture (10−100μl) was heated at 95°C for 20 min and annealed for 1.5h at 42°C in 250mM NaCl/5mM $MgCl_2$/5mM Tris-HCl, pH 8.0 Heteroduplexed DNA was precipitated with ethanol, washed and then resuspended in distilled water.

For the experiment of Fig. 6A, a 98 bp region of the s15 chorion gene promoter was amplified symmetrically using two primers flanking that region. A portion of the product was used to amplify asymmetrically the sense strand that includes the wild type 34C sequence (subsequent experiments showed that simple denaturation of the symmetrically amplified product, chilling and application to the ice-cold membrane is also satisfactory). The final product was electrophoresed in a 3% NuSieve/1% ME agarose 1× TBE gel until clear separation of the main band from a minor background was achieved . Then a slit in the gel was cut with a razor blade just ahead of the band of interest, and a piece of Hybond M-G paper(Amersham), prewet in TBE buffer, was inserted. Another strip of DEAE cellulose may be placed just behind the band to prevent contamination with larger molecules. Electrophoresis was then resumed for a few more minutes resulting in quantitative retention of DNA (unpublished results). The strip was removed, dried to immobilize the DNA, rinsed in double distilled water (twice), and eventually dehydrated in 96% ethanol. The dried paper was then placed inside a 500 μl Eppendorf tube and covered with about 100 μl 1× annealing buffer (see above). End labelled 31T or 31G oligonucleotide probe was then added and left to anneal with the paper-immobilized DNA overnight, as described above. The $KMnO_4$ reaction was performed as described in the next section.

Potassium permanganate reaction

$KMnO_4$ was prepared as a stock 100mM solution. A 5M Me_4NCl stock was prepared by dissolving in water the contents of a vial of solid reagent (Aldrich) once after opening. No correction was made for hydration of the solid prior to making the stock solution.

DNA probes were mixed with 6 μl of 5M Me_4NCl in a total volume of 10 μl and left 10 min at room temperature to equilibrate. Then 10 μl 0.2mM $KMnO_4$/3M Me_4NCl was added (from a mixture prepared by adding 1 μl of 100mM $KMnO_4$ in 500 μl 3M Me_4NCl). The reaction was left to proceed for 30 min at 20°C, spiked with 5 μl of $KMnO_4$/3M Me_4NCl and left to proceed for another 30 min. The reaction was stopped by adding 200 μl of a solution containing 0.3M sodium acetate, 0.1M Na_2EDTA and 25 μg/ml tRNA, and the DNA was precipitated with ethanol twice, washed with 70% (vol/vol) ethanol and dried. Ethanol precipitation is necessary to remove Me_4NCl since it interferes with electrophoresis.

When the DNA was immobilized on Hybond M-G, the reaction started by immersing the paper, still wet from the previous annealing step, in 250 μl of 3M Me_4NCl inside a 500 μl Eppendorf tube and then adding 250 μl of 0.2 mM $KMnO_4$/3M Me_4NCl (see above). The reaction was left to proceed for 30 min at 20°C and then the solution was replaced with freshly prepared one and left to proceed for another 30 min. The reaction was stopped by simply removing the paper and rinsing it with double distilled water. After another round of rinsing and dehydration in ethanol, the paper was immersed in 1M piperidine (100 μl) and incubated as described below. It is important that the paper remains wet after the annealing step, since drying immobilizes the probe and causes increased intensity of background bands.

Hydroxylamine reaction

Conditions were essentially as in Cotton *et al.* (4), except that for oligonucleotide probes Tris-HCl pH 7.4 was included at 50mM final concentration, and the reaction was performed at 20°C. When Et_4NCl was included in the reaction, hydroxylamine hydrochloride was dissolved in 2M Et_4NCl instead of water and the pH adjusted to 6.0 with diethylamine. From this stock solution, 20 μl were added to 6 μl containing DNA in 2M Et_4NC1, and the reaction was left to proceed for 2−4 hrs at 20°C. The hydroxylamine/Et_4NCl solution was prepared fresh each week. The Et_4NCl was prepared as a 4M stock solution, while, in comparable experiments, propylammonium chloride was prepared as a 2.6M stock solution in water, once after opening. Again, no correction was made for the hydration of the solid. For Hybond M-G immobilized DNA, reactions were performed as described above with the exception that they were initiated by immersing the strip in 400μl of hydroxylamine/Et_4NC1 solution (100μl 2M Et_4NCl + 300 μl hydroxylamine/Et_4NCl) and were left to proceed for 2 h.

Piperidine cleavage

Chemical cleavage of DNA at C and T modified bases, reacted with hydroxylamine or $KMnO_4$, was done by incubating in 1M piperidine (50μl) at 90°C for 30 min (20). Piperidine was removed by repeated cycles of freezing ahd lyophilization.

Analysis of cleavage products

Samples were taken up in 10 μl of 80% (vol/vol) formamide/0.1% bromophenol blue/0.1% xylene cyanol FF/35mM Na_2EDTA pH 8.0, heated at 90°C for 4 min, placed on ice and then electrophoresed on denaturing urea gels. For quantitation, equal amounts of radioactivity were loaded in each lane and gels were exposed to film without screen. Several exposures were scanned in an LKB ULTROSCAN XL Laser Densitometer, and paired nucleotides located far from the mismatch, at the ends of the molecules, were used as internal control.

RESULTS

Test DNAs

Oligonucleotide probes are valuable in genetic studies, especially for diagnostic purposes, yet are notorious for high background in chemical cleavage reactions. Therefore, we have used oligonucleotides as a stringent system for developing our techniques. These oligonucleotides were built from three 34-mers and three 31-mers, corresponding to the sense and antisense strands, respectively, both wild type and mutated, of the *Drosophila s15* chorion gene promoter (see Materials and Methods). All mutations, in both strands, were in the same , −98 relative to the cap site of the *s15* gene (11). The oligonucleotides were designated according to the strand and choice of nucleotide at that position as 34C (wild type), 34T, 34A, 31G (wild type), 31A or 31T; labelled strand was listed first. Appropriate choice of a pair of oligonucleotides permitted formation of the homoduplex (34C/31G) and six heteroduplex (CA, CT, TG, TT, AG,AA) combinations. Additional 63 and 76 bp regions of *s15* DNA were also used as test sequences (see Materials and Methods).

To test whether the method is capable of accurately detecting single base pair changes in whole human genomic DNA, we took advantage of two known T → C polymorphisms at D3S14 locus(14). By direct sequencing of genomic DNA(15), we had determined the DNA sequence flanking the polymorphic sites and detected the single base substitution (V.Stanton and H. Aburatani, unpublished data). We amplified the target sequences from whole genomic DNA by PCR and used the products to construct long (210 or 351 bp) homoduplexes and heteroduplexes (see Materials and Methods). Finally, in an exact analog to experiments using oligonucleotides to probe for mutations or polymorphisms in genomic DNA, we used the *s15* oligonucleotides to construct heteroduplex or homoduplex combinations with a PCR-amplified, 98 nucleotide segment of the *s15* promoter.

Detection of T mismatches

We used $KMnO_4$ for detection of T mismatches, because at low concentrations it modifies preferentially T residues in single stranded DNA (T>C>>G>A); it reacts with double stranded DNA at a much slower rate (16). $KMnO_4$ alone, probably because it is a strong oxidant, did not discriminate adequately between a heteroduplex (34T/31G) and a homoduplex (34T/31A): It showed a relative ratio of 1.2 for cleavage of the two DNAs at that site (Fig.1, lanes 3 and 4). However, when the reaction was performed in the presence of 3M Me_4NCl, the ratio was increased approximately 4-fold (Fig. 1, lanes 1 and 2), resulting in excellent discrimination, at least as good as that achieved with osmium tetroxide (Fig. 1, lanes 5 and 6).

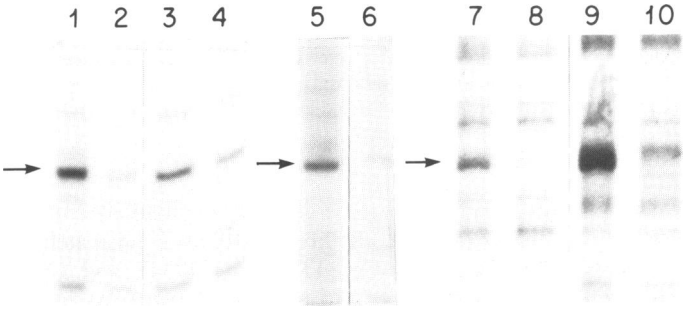

Figure 1. Effect of Me_4NCl on the $KMnO_4$ reaction. Incubations were as described in Materials and Methods, with or without 3M Me_4NCl. *Lanes 1 and 2*: 34T/31G heteroduplex (1) and 34T/31A homoduplex (2), incubated with $KMnO_4$/Me_4NCl. *Lanes 3 and 4*: the same heteroduplex (3) and homoduplex (4) oligonucleotides incubated with $KMnO_4$ alone. *Lanes 5 and 6*: the same heteroduplex (5) and homoduplex (6) oligonucleotides incubated with osmium tetroxide. *Lanes 7 to 10*: The 351 bp heteroduplex (7,9) or homoduplex (8,10) fragment, incubated with $KMnO_4$ alone (7,8), or $KMnO_4$/3M Me_4NCl (9,10). Arrows indicate the base pair of interest.

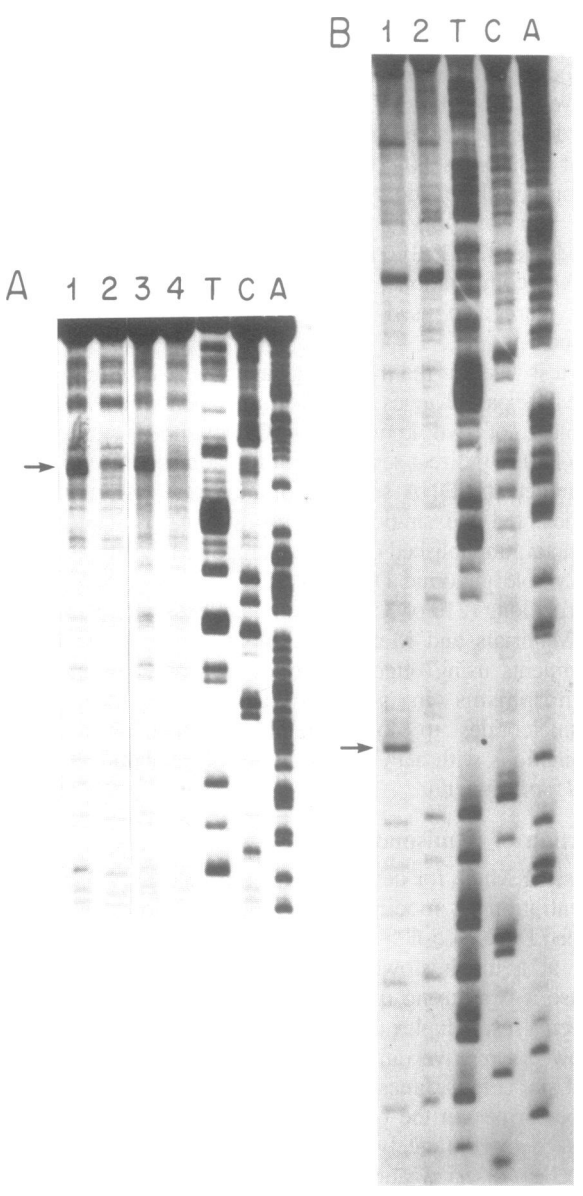

Figure 2. Detection of two T → C polymorphisms using whole genomic DNA and PCR amplification (see the Text). *A*: A 351 bp fragment, either as heteroduplex with a TG mismatch (lanes 1,3) or as homoduplex control (lanes 2,4), incubated with either $KMnO_4$/3M Me_4NCl (lanes 1,2) or osmium tetroxide (lanes 3,4). *B*: A different 210 bp fragment, either as heteroduplex with a TG mismatch (lane 1) or as homoduplex control (lane 2). In both panels, lanes marked T, C ,A show Maxam-Gilbert sequence ladders of the end-labelled probes. Arrows indicate the nucleotides of interest.

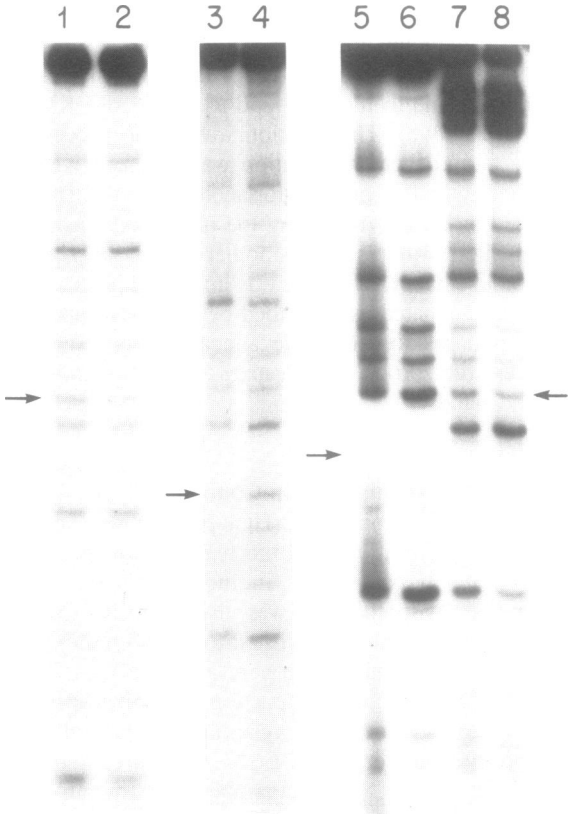

Figure 3. Specificity of the permanganate reaction. *Lanes 1–4:* the 34C/31T heteroduplex (lanes 1,4) and the 34C/31G homoduplex (lanes 2,3), incubated with $KMnO_4$/3M Me_4NCl (lanes 1,2) or osmium tetroxide (lanes 3,4). *Lanes* 5–8: $KMnO_4$/Me_4NCl reactions using the 34A/31T homoduplex (lane 5), the 34A/31G heteroduplex (lane 6), the 31G/34C homoduplex (lane 7) and the 31G/34T heteroduplex (lane 8). Arrows indicate the base pair of interest.

This phenomenon appears to be general: qualitatively similar results were obtained with all three possible T mismatches, including 34T/31G, 31T/34C, 34T/31T, 31T/34T and a TT mismatch in a 63bp region of s15 (data not shown). As in the case of osmium tetroxide (4), the reactivity of T in TT mismatches was lower than in TG or TC mismatches, perhaps because of stacking or even base pairing of TT duplexes (17). Discrimination of T mismatches was also improved by 2M Et_4NCl, but not 0.5 to 1M NaCl; no specific cleavage was seen in heteroduplex controls without incubation, with Me_4NCl but without $KMnO_4$, or without piperidine (data not shown).

Excellent discrimination was also observed with T mismatches in long DNA fragments rather than oligonucleotides. Genomic DNAs, from two individuals homozygous for either the T or the C polymorphisms at the D3S14 locus, were used to amplify by PCR corresponding fragments (210 and 351 bp). In both cases, the primer used to amplify the strand carrying the T polymorphism was ^{32}P end labelled. Labelled homoduplexes were used directly, or were mixed with excess unlabelled products carrying the C polymorphism, denatured and reannealed, yielding T labelled TG heteroduplexes (see Materials and Methods). With these DNA fragments, $KMnO_4$ plus 3M Me_4NCl substantially enhanced discrimination of the TG mismatch over the $KMnO_4$ alone (Fig. 1, lanes 7–10). The mismatched T was the only band that was enhanced relative to the homoduplex (Figs.2A and 2B, lane 1 vs 2). Discrimination was at least as good as with osmium tetroxide (Fig.2A, lanes 3 and 4).

The specificity of the T mismatch reaction

The specificity of mismatch detection by $KMnO_4$ in the presence of Me_4NCl was tested with heteroduplex oligonucleotides bearing CA, CT, GA and AG mismatches, in comparison with the corresponding homoduplexes. The C mismatches were modified at a ratio of 2.0 compared to the homoduplex (Fig.3, lanes 1 and 2); this moderate enhancement was expected from the known specificity of $KMnO_4$ in single-stranded DNA (T > C > > G > A), and was also seen with osmium tetroxide (Fig.3, lanes 3 and 4). No preferential cleavage of the G or A mismatches was observed, as expected (Fig.3, lanes 5–8). Similar results were obtained with an AC mismatch in a 63 bp

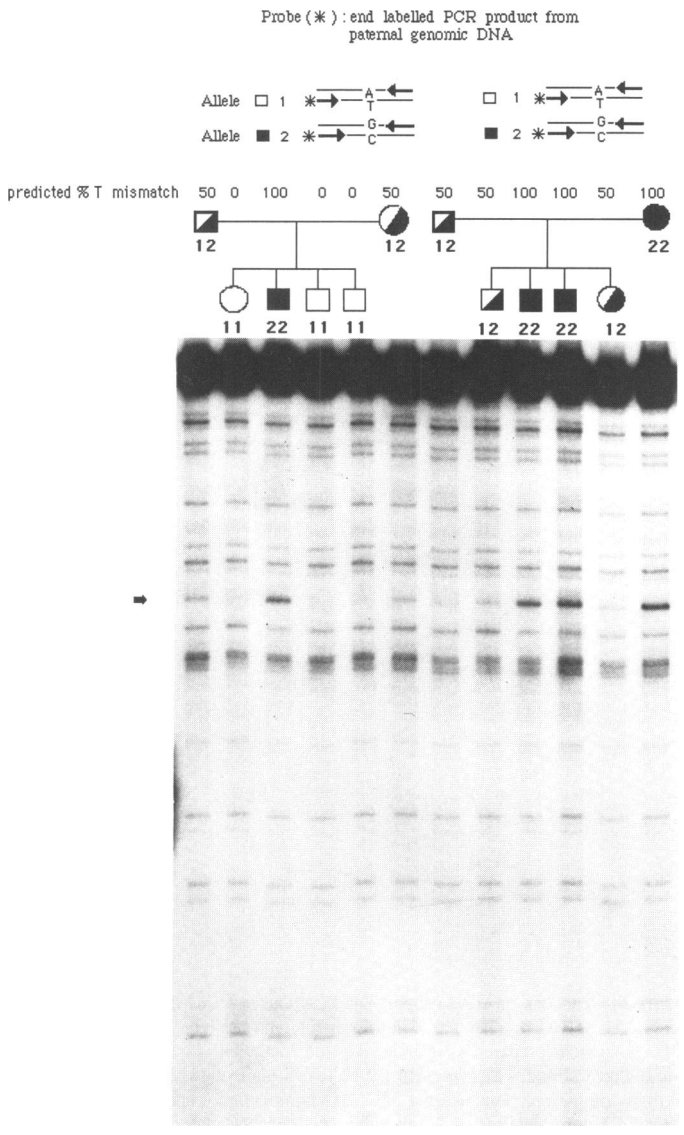

Figure 4. Inheritance of a T vs. C polymorphism in two unrelated families, as followed by the $KMnO)_4/Me_4Cl$ reaction for T mismatch detection. The probe in each case was end labelled paternal amplified DNA, which was heterozygous for the two alleles (see Text). The alleles are indicated both by numbers and open (allele1,T) or filled boxes (allele 2,C). In the diagram, arrows indicate the PCR primers and asterisks ^{32}P label. The predicted percentages of T mismatches are listed above the pedigrees, and are seen to be in excellent agreement with the intensities of the signal in the autoradiogram, at the position of interest (arrow).

heteroduplex, and with GG and AA mismatches in a 76 bp heteroduplex (data not shown).

With both osmium tetroxide and hydroxylamine, the phenomenon of propagation has been encountered (4,18), whereby paired bases near a mismatch become reactive. We have noted sensitization of selected bases either adjacent to or as far as 5 positions away from the nearest mismatch, in $KMnO_4$ reactions using a 63 bp heteroduplex, in which three mismatches were present within 20 bp, destabilizing that region (data not shown). Despite such possible secondary modifications in some situations, the primary mismatch is usually distinguishable by its intensity and responsiveness to Me_4Cl (see also below).

The workable specificity of the reaction for T mismatch detection was best documented by its ability to follow the Mendelian inheritance of a genetic polymorphism in two different families (Fig. 4). In each case, the probe was PCR amplified, end labelled paternal DNA. Both fathers were heterozygous (alleles 1 and 2) and thus, each probe contained a 1:1 mixture of labelled strands bearing T or C respectively, in a single polymorphic site. The probe was mixed and annealed with 20 fold excess of unlabelled amplified DNA from each family member, and the $KMnO_4/Me_4NCl$ reaction was used to detect possible mismatches in the annealed product.

It was predicted that at the polymorphic site, three different patterns would be detectable. If the family member tested was homozygous for the T allele (1,1), the T strands of the probe should be homoduplexed, and no signal would be expected above background (the background would be caused by weak modification of the CA mismatch). At the other extreme, if the family member was homozygous for the C allele (2,2), the T strands of the probe would be present as TG heteroduplexes, resulting in a strong signal. Finally, if the family member was heterozygous (1,2), some of the T strands should be heteroduplexed and some should be homoduplexed, resulting in an intermediate signal. This is precisely what was observed (Fig.4).

Detection of C mismatches

Hydroxylamine is also known to modify single stranded DNA faster than double stranded DNA (16), and has been used to detect mismatched cytosines in long DNA fragments (4). With our test oligonucleotides, optimal conditions were slightly different than previously reported (4): 50 mM Tris-HCl pH 7.4 and incubation at 18−20°C (see also Materials and Methods). Despite the optimization, with hydroxylamine alone we were only able to achieve a discrimination ratio of 2 for the 34C/31A and 34C/31T heteroduplexes relative to the 34C/31G homoduplex (see Fig.5, lanes 1 and 7 for the CT heteroduplex and the CG homoduplex, respectively). This low signal-to-noise ratio may be due to the high reactivity of short duplexes, or to influences of the sequence context (19). However, we were able to increase the ratio up to approximately 6, by including 2.0 to 2.5 M Et_4NCl in the reaction mixture. Figure 5 documents that the higher ratio results from more efficient modification of the mismatched C with increasing concentrations of Et_4NCl, while the reactivity of homoduplexed C bases remains constant. Only at 4M Et_4NCl, as the strands dissociate (7), do the homoduplexed cytosines become highly reactive, and the preferential modification of the mismatch disappears. Secondary reactivity of a C immediately 5′ to the mismatch was observed (as in the standard hydroxylamine reaction; 4), but the mismatch could be easily distinguished by its intensity and its relative extinction when the concentration of Et_4NCl is raised to the level of making homoduplexed C bases reactive.

Reactions on solid supports

We have shown that our conditions for both the potassium permanganate and the hydroxylamine reactions are compatible with the use of Hybond M-G (Amersham) chemically modified paper (10). This paper permits immobilized DNA to be modified reliably by certain reagents, and to be eluted subsequently at 90°C with piperidine, which also cleaves a certain proportion of the modified bases. Thus, use of this paper is known to greatly simplify Maxam-Gilbert sequencing by avoiding the time consuming ethanol precipitation steps, eliminating all traces of salt from the samples, permitting simultaneous modification of many DNA samples and offering the potential for automation. Evidently, the same advantages are applicable to mismatch detection.

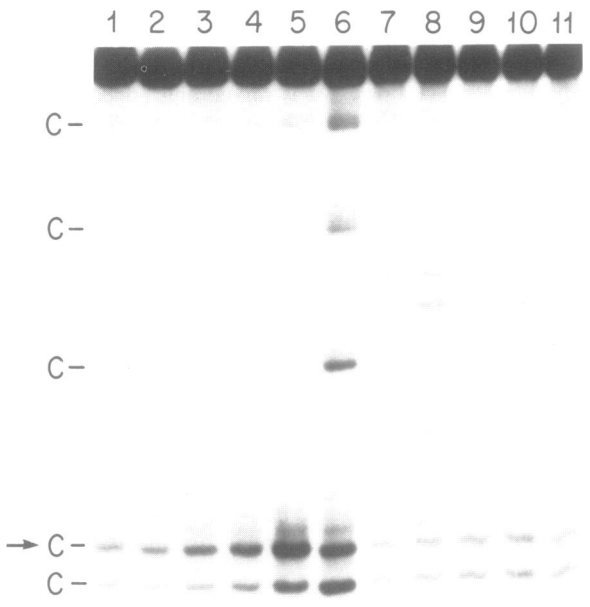

Figure 5. Effect of Et_4NCl on the hydroxylamine reaction. The 34C/31T heteroduplex (lanes 1–6) and the 34C/31G homoduplex (lanes 7–11) were incubated with 2M hydroxylamine at 18°C, in the presence of the following Et_4NCl concentrations: zero (lanes 1,7), 0.1M (lanes 2,8), 0.5M (lanes 3,9), 1M (lanes 4,10), 2M (lanes 5,10) and 4M (lane 6). The C bases are indicated, and arrows mark the nucleotide of interest.

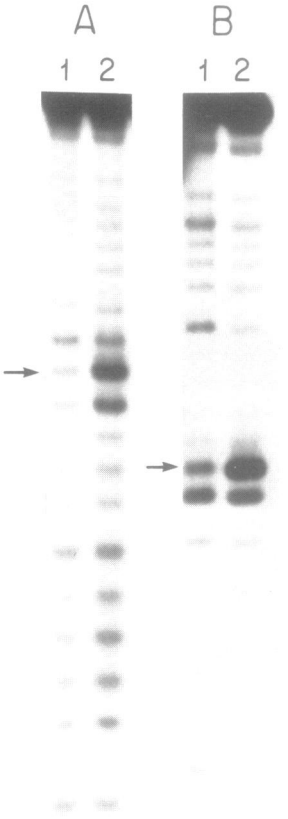

Figure 6: Reactions on solid support. *A*: Reactions with $KMnO_4$/3M Me_4NCl, using a 31 bp GC homoduplex control (lane 1) and a 31 bp heteroduplex with a TC mismatch (prepared as in Materials and Methods). The unlabelled paper-immobilized strand was generated by PCR (98 nucleotides, see the text). *B* : Hydroxylamine reactions using a 34C/31G homoduplex control (lane 1) and a 34C/31T heteroduplex (lane 2), prepared as described in text. Note the partial enchancment of the 5′ neighbouring T of the T mismatch and the reactivity of the 5 neighbouring C of the C mismatch position. Arrows indicate the nucleotide of interest.

A typical T mismatch detection experiment of this kind is illustrated in Fig. 6A. The wild type promoter region of the *s15* gene was amplified symmetrically with PCR, yielding a 98 bp product, and the sense strand (which includes the 34C sequence) was then amplified asymmetrically with the appropriate primer (see Materials and Methods). The products were separated in an agarose gel, and the single-stranded sense band was electrophoretically transferred to a piece of Hybond M-G paper, which was placed in front of the band in a slit. The paper was removed, dried to immobilize the DNA, rinsed, dehydrated in ethanol and placed in annealing buffer (see Materials and Methods). End labelled 31T or 31G oligonucleotide was then added and annealed to the immobilized DNA. The wet paper was transferred into the $KMnO_4$/Me_4NCl reaction solution incubated, rinsed briefly, dehydrated in ethanol and treated with piperidine, as usual. An experiment for C mismatch detection using immobilized 34C/31G or 34C/31C oligonucleotides and hydroxylamine is illustrated in Fig.6B. In general, discrimination of mismatches proved to be better with Hybond M-G immobilized DNA than with DNA in solution. In Fig. 6, note the strong enhancement of each mismatch which is easily discriminated from weaker secondary bands due to either propagation (see the homoduplexed T 5′ to the T mismatch position in Fig.6A) or to unusual reactivity (see the homoduplexed C 5′ to the C mismatch position in Fig.6B).

DISCUSSION

We have identified conditions, using tetramethylammonium chloride, that make potassium permanganate suitable for detection of mismatched thymines. The specificity of the reaction is comparable to that of osmium tetroxide (4), which is much more hazardous. As with osmium tetroxide (4), mismatched cytosines also react, albeit at a slower rate. Concomitant application of the C-specific hydroxylamine reaction unambiguously resolves T and C mismatches. Modification of the mismatched C base by hydroxylamine is enhanced in the presence of tetraethylammonium chloride. If these two reactions are applied to both DNA strands, they are capable of detecting all point mutations or genetic polymorphisms. Propagation of the disrupted DNA structure results in weaker signals from T or C homoduplexed bases immediately adjacent to a mismatch, or even several positions away if that region of DNA contains multiple mismatches and is consequently destabilized. In some cases, these false secondary signals may introduce ambiguities. However, they may also be helpful in revealing nearby hidden mismatches (e.g.

A or G), which are then identified with additional experiments. The methods we propose should be valuable for rapid screening purposes, followed when appropriate by more detailed studies, e.g. PCR-aided definitive determination of the mismatched sequence. They should be applicable to detection of small insertions and deletions as well as point mutations (20,21), and when used on paper-immobilized DNA they should be amenable to automation. A potentially major application would be to screen random cloned probes for point polymorphisms .

The mechanisms of tetralkylammonium enhancement may be different for T and C modifications. In the case of potassium permanganate a weaker interaction of Me_4NCl with TC, TG and TT mismatched bases (as compared to TA base pair) could explain the observed enchacement of reactivity. In the case of the C reactions, we have observed enhancement both with Et_4NCl, and with propylammonium bromide up to concentrations that cause strand dissociation (data not shown): for hydroxylamine, but not potassium permanganate, increased hydrophobicity of the environment (6) may be crucial. An alternative explanation is the lower ability of ethyl- and propyl-ammonium to screen the phosphate charges of DNA. Further experimentation is needed to distinguish among the various possibilities. In either case, sequence dependence of the effect of tetralkylammonium salts on the C reaction is expected.

We first tested potassium permanganate and hydroxylamine for detection of T or C mismatches, because they were known to react faster with single stranded as compared to double stranded DNA (16). However, even if this property is necessary, it appears not to be sufficient: it has been reported, for example, that diethyl pyrocarbonate, which is known to react with adenines preferentially in single stranded conformation, is unable to modify mismatched adenines (19); even KMnO4 alone does not discriminate very well between heteroduplex and homoduplex T's. Crystallographic and NMR studies (22,23), enzymatic cleavage and electrophoretic analysis (19) all suggest that mismatching is not very disruptive of the double helical structure, but can disrupt locally stacking. Indeed, all three chemicals which are now known to be useful as mismatch detectors (osmium tetroxide, hydroxylamine and now potassium permanganate) react with the 5,6 double bond, and therefore can be expected to 'sense' local disruptions in stacking. This may be the most pertinent property for mismatch detectors.

In a recent study, Bhattacharyya and Lilley (19) have demonstrated a sequence context dependence in the reactivity of mismatched cytosines to hydroxylamine. For all six kinds of C mismatches they studied (two of each CT, CA and CC), only those that were flanked by two purines were reactive, whereas mismatches flanked by two pyrimidines were completely inert. Interestingly, in the latter case the reactivity of the CC mismatch was restored by raising the pH to 7.0, probably through the induction of a local structural rearrangement. Similarly, position dependence has been demonstrated for osmium tetroxide. We have not noted major position dependence with potassium permanganate in the presence of Me_4NCl, for five different sequence contexts, but it is premature to judge the generality of this observation. Conceivably, in some cases tetralkylammonium salts may help overcome position dependence of reactivity, further improving mismatch detection.

In our experience, major savings in effort are possible when mismatch detection is performed on DNA immobilized on Hybond M-G. Together with the convenience of using $KMnO_4$ rather than osmium tetroxide for T mismatches, this technical improvement should greatly facilitate the detection of point mutations and genetic polymorphisms. The ease of the procedure should permit both the T and the C reactions to be performed in parallel, for both strands, maximizing the detection of mismatches and minimizing the misinterpretation of propagation artifacts.

Oligonucleotide probes, for which our reaction conditions are particularly suitable, are ideal for diagnosis of diseases well characterized at the molecular level (e.g. thalassaemias, where novel mutations revealed by sequencing are rare; 23), or polymorphisms already characterized as genetic markers. Alternatively, PCR can be used to amplify and label genomic sequences to be screened for mismatches, thus overcoming problems of sensitivity and specificity that are often encountered when working directly with total genomic DNA. Similar PCR methodologies have been published by others recently (20,21). This approach has the advantage of permitting the scanning for mutations in relatively long DNA segments. As we have demonstrated, the technique is quantitative enough for discrimination of heterozygotes from homozygotes, thus permitting the Mendelian analysis of mutations or polymorphisms.

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