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Reactivity of potassium permanganate and tetraethylammonium chloride with mismatched bases and a simple mutation detection protocol

A. Lambrinakos, K. E. Humphrey, J. J. Babon, T. P. Ellis and R. G. H. Cotton*

Mutation Research Centre, 7th Floor, Daly Wing, 41 Victoria Parade, Fitzroy, Melbourne, Victoria 3065, Australia

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ABSTRACT

Many mutation detection techniques rely upon recognition of mismatched base pairs in DNA heteroduplexes. Potassium permanganate in combination with tetraethylammonium chloride (TEAC) is capable of chemically modifying mismatched thymidine residues. The DNA strand can then be cleaved at that point by treatment with piperidine. The reactivity of potassium permanganate ($KMnO_4$) in TEAC toward mismatches was investigated in 29 different mutations, representing 58 mismatched base pairs and 116 mismatched bases. All mismatched thymidine residues were modified by $KMnO_4$/TEAC with the majority of these showing strong reactivity. $KMnO_4$/TEAC was also able to modify many mismatched guanosine and cytidine residues, as well as matched guanosine, cytidine and thymidine residues adjacent to, or nearby, mismatched base pairs. Previous techniques using osmium tetroxide (OsO_4) to modify mismatched thymidine residues have been limited by the apparent lack of reactivity of a third of all T/G mismatches. $KMnO_4$/TEAC showed no such phenomenon. In this series, all 29 mutations were detected by $KMnO_4$/TEAC treatment. The latest development of the Single Tube Chemical Cleavage of Mismatch Method detects both thymidine and cytidine mismatches by $KMnO_4$/TEAC and hydroxylamine (NH_2OH) in a single tube without a clean-up step in between the two reactions. This technique saves time and material without disrupting the sensitivity and efficiency of either reaction.

INTRODUCTION

Mismatched bases have been shown to be reactive with various chemicals. Mismatched guanine and thymine bases are reactive with a carbodiimide (1) and mismatched cytosine and thymine bases are reactive with hydroxylamine (NH_2OH) and osmium tetroxide (OsO_4), respectively (2). Potassium permanganate ($KMnO_4$) in association with either tetramethylammonium chloride (TMAC) (3) or tetraethylammonium chloride (TEAC) (4) reacts with mismatched thymine bases and has been suggested as a replacement for the toxic OsO_4.

This reactivity of mismatched bases has been exploited to develop protocols for mutation detection after hybridisation of mutant and wild-type DNA (or RNA). These include the Carbodiimide method (1) and the Chemical Cleavage of Mismatch (CCM) method (reviewed in 2,5). This paper examines the reaction of $KMnO_4$ on 58 different mismatches of all types and represents the reactivity of 116 mismatched bases and the surrounding sequence with $KMnO_4$ and TEAC employing the CCM methodology.

MATERIALS AND METHODS

PCR

The reactivity of $KMnO_4$ (Sigma, USA) in TEAC (Sigma, USA) was studied with 29 different cloned mouse-β-globin promoter mutants (6) kindly provided by Dr R. Myers. Plasmids containing wild-type and mutant DNA were amplified using fluorescent or biotin labelled primers. The primers were 5′-labelled with the biotin or the fluorophore (6-FAM for the 5′ primer, HEX for the 3′ primer). The sequence of the primers used are as follows (6): 5′ primer, 5′-GCACGCGCTGGACGCGCAT; 3′ primer, 5′-AGGTGCCCTTGAGGCTGTCC. The PCR conditions were an initial denaturation at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 1 min and a further extension at 72°C for 10 min. This generated 555 bp products. The DNA labelled with biotin is represented by B and those labelled with fluorophore designated F. The mismatch base on the fluorescent labelled strand is represented by an asterisk in figures and tables.

Chemical reactions

Separate reactions. This paper utilises and examines in detail a modification of the original CCM procedure (2) that replaces OsO_4 with $KMnO_4$ and TEAC (4).

DNA heteroduplexes were formed by adding ~20 µl of 100 ng of wild-type and 100 ng of mutant DNA to 20 µl of 2× annealing buffer (1.2 M NaCl, 12 mM Tris–HCl pH 7.5, 14 mM $MgCl_2$) (6). The solution was then boiled for 5 min, annealed for 80 min at 65°C and allowed to cool slowly to room temperature overnight. The biotinylated DNA was either attached to streptavidin-coated magnetic beads (Dynabeads M-280; Dynal, Norway) (7) or the reactions were performed in aqueous solution. The binding of the biotinylated DNA to the streptavidin-coated magnetic beads required washing the streptavidin beads (3 µl per reaction) with Bind and Wash buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0, 2 M NaCl). The streptavidin beads are resuspended in Bind and Wash (3 µl per reaction). Aliquots of 3 µl of the beads in solution are added to each heteroduplex and incubated with shaking at room temperature. The supernatant is removed and the beads are washed with 20 µl of TE (10 mM Tris, 1 mM EDTA) buffer.

*To whom correspondence should be addressed. Tel: +61 3 9288 2980; Fax: +61 3 9288 2989; Email: cotton@ariel.ucs.unimelb.edu.au

NH_2OH (BDH, UK) and OsO_4 (Aldrich, USA) DNA modifications were performed as reported previously (2). Briefly, 75–100 ng of biotinylated DNA heteroduplexes bound to the streptavidin-coated magnetic beads in TE buffer were incubated in 20 µl of 4.2 M NH_2OH solution for 30 min at 37°C. For the OsO_4 reactions 2.5 µl of OsO_4 buffer (10 mM EDTA, 100 mM Tris–HCl pH 7.7, 15% pyridine) and 15 µl of 0.8% OsO_4 solution is added to the heteroduplexes and incubated for 5 min at 37°C. The NH_2OH and OsO_4 incubations are terminated by pelleting the beads and then washing twice with 50 µl TE buffer.

The procedure incorporating $KMnO_4$ and TEAC was carried out as described by Roberts *et al*. (4). Briefly, 75–100 ng of heteroduplexes were incubated in 20 µl of 1 mM $KMnO_4$/3 M TEAC for 45 min at 25°C. The reactions were stopped by pelleting the beads, removing the supernatant and subsequent washing of the DNA with TE buffer. If the DNA was aqueous rather than attached to the magnetic beads the $KMnO_4$ incubation time was 5 min and the reactions were stopped with 250 µl of stop buffer (0.3 M sodium acetate pH 5.2, 0.1 mM EDTA pH 8.0, 25 µg/ml tRNA solution) (2) and 750 µl of ethanol and the DNA precipitated. In all cases the modified bases were then cleaved by treatment with 10% aqueous piperidine (Fluka, Switzerland) at 90°C for 30 min. This reaction was stopped by ethanol precipitation and the fluorescent reaction products were separated on a denaturing 4.25% polyacrylamide gel using an ABI-377 DNA sequencer (Perkin Elmer, CA).

Sequential, single tube CCM reactions. In order to attempt to simplify the CCM procedure, the same sample of DNA in a single tube was modified with two sets of reagents, first $KMnO_4$/TEAC to modify mismatched thymine bases and secondly NH_2OH to modify mismatched cytosine bases. DNA heteroduplexes (150–200 ng) were attached to the Dynabeads first incubated with $KMnO_4$/TEAC under standard conditions for 45 min at 25°C followed by an equal volume of 11.5 M hydroxylamine hydrochloride (pH to 6.0 with diethylamine) and the incubation continued for a further 40 min at 25°C. If the DNA was aqueous rather than attached to a solid phase then the $KMnO_4$ incubation was for only 5 min. Piperidine cleavage was identical to that described above.

Identifying modified nucleotides. The actual nucleotides modified by $KMnO_4$ in selected samples were identified using shorter 148 bp fragments of the same mutants. These were amplified using the same PCR conditions and 5′ primer as described above and a 3′ primer of sequence CACAACTATGTCAGAAGC (either biotinylated or HEX labelled). To distinguish mismatches, one strand in the heteroduplex was fluorescently labelled while the other was biotinylated. The reactions were electrophoresed on the ABI-377 sequencer adjacent to A, C, T and G dye primer sequencing tracts generated by sequencing the 148 bp wild-type fragment with the same 5′ and 3′ primers used to amplify the fragment and a Dye primer sequencing kit (Perkin Elmer, CA). The sequencing fragment of identical size represents the nucleotide immediately 5′ to the site of modification by $KMnO_4$ as the modified nucleotide itself is destroyed during the piperidine treatment.

RESULTS AND DISCUSSION

The reactivity of $KMnO_4$ in TEAC toward mismatched bases was investigated in 58 different mismatched base pairs representing 116 mismatched bases as summarised in Table 1. Solid phase

Table 1. The reactivity of $KMnO_4$ in TEAC with 29 different Myer's mutants representing 58 different mismatches and 116 mismatched bases

Class I: G↔A, C↔T mutations lead to TG, CA mismatches		Class II: G↔T, C↔A mutations lead to TC, GA mismatches	
WT/-27G	H H	WT/-43A	M N
-TATAAGG	-TATGAGG	-GAGCCAG	-GAGACAG
ATACTCC-	ATATTCC-	CTCTGTC-	CTCGGTC-
H	M	H	N
WT/-39A	N N	WT/-89A	N N
-CAGGGCA	-CAGAGCA	-ACACCCT	-ACAACCT
GTCTCGT-	GTCCCGT-	TGTTGGA-	TGTGGGA-
H	N	L	N
WT/-78A	N N	WT/-35T	N H
-AGGACCA	-AGGGCCA	-GCAGAGC	-GCATAGC
TCCCGGT-	TCCTGGT-	CGTATCG-	CGTCTCG-
N	M	N	H
WT/-37T	N H	WT/-33T	N H
-GGGCAGA	-GGGTAGA	-AGAGCAT	-AGATCAT
CCCATCT-	CCCGTCT-	TCTAGTA-	TCTCGTA-
L	M	N	M
WT/-25A	H H	WT/-13G	N H
-TAAAGTG	-TAAGGTG	-GGAGCAG	-GGATCAG
ATTCCAC-	ATTTCAC-	CCTAGTC-	CCTCGTC-
L	M	N	H
WT/-57G	H H	WT/-54T	N H
-GATAGAG	-GATGGAG	-AGAGAGG	-AGATAGG
CTACCTC-	CTATCTC-	TCTATCC-	TCTCTCC-
N	H	L	H
WT/-14G	H H		
-AGGATCA	-AGGGTCA		
TCCCAGT-	TCCTAGT-		
L	H		
WT/-18C	H N		
-AGGTAGG	-AGGCAGG		
TCCGTCC-	TCCATCC-		
H	H		
WT/-21G	L H		
-GTGAGGT	-GTGGGGT		
CACCCCA-	CACTCCA-		
N	H		
WT/-36G	N N		
-GGCAGAG	-GGCGGAG		
CCGCCTC-	CCGTCTC-		
M	M		
WT/-26G	H M		
-ATAAGGT	-ATAGGGT		
TATCCCA-	TATTCCA-		
H	H		
WT/-54A	M N		
-AGAGAGG	-AGAAAGG		
TCTTTCC-	TCTCTCC-		
H	H		
WT/-82G	N N		
-GGTGAGG	-GGTAAGG		
CCATTCC-	CCACTCC-		
L	M		
WT/-11G	H N		
-ATCGGTT	-ATCAGTT		
TAGTCAA-	TAGCCAA-		
H	N		
WT/-85A	H H		
-CCTGGTA	-CCTAGTA		
GGATCAT-	GGACCAT-		
M	N		
WT/-4T	H L		
-GCTCCTC	-GCTTCTC		
CGAAGAG-	CGAGGAG-		
N	H		
WT/-68C	H L		
-TGCTCAC	-TGCCCAC		
ACGGGTG-	ACGAGTG-		
N	N		
WT/-74G	H H		
-CCAGTCT	-CCAATCT		
GGTTAGA-	GGTCAGA-		
H	L		
Class III C↔G mutations lead to GG, CC mismatches		**Class IV** A↔T mutations lead to AA, TT mismatches	
WT/-42G	H L	WT/-13A	H N
-AGCCAGG	-AGCGAGG	-GGATCAG	-GGAACAG
TCGCTCC-	TCGGTCC-	CCTTGTC-	CCTAGTC-
H	L	H	N
WT/-76G	H N	WT/-2A	H N
-GGCCAAT	-GGCGAAT	-TCCTCAC	-TCCACAC
CCGCTTA-	CCGGTTA-	AGGTGTG-	AGGAGTG-
H	L	H	N
WT/-12G	H N		
-GATCAGT	-GATGAGT		
CTACTCA-	CTAGTCA-		
H	L		

The classes are categorised according to base changes, which results in two different types of mismatches. For example, a C→G base change or vice versa leads to a CC and a GG mismatch (Class III). L, low reactivity; M, medium rectivity; H, high reactivity; N, no reactivity; **T**, reactivity of the mismatch may be due to an adjacent matched T, which is in bold.

Table 2. A summary of the different types of mismatches and their surrounding bases reactivity with $KMnO_4$

Mismatch	No Reactivity	Weak Reactivity	Intermediate Reactivity	Strong Reactivity	Total Mismatches	% mismatches detected in total	% detected with no adjacent T.
C*/A	8	4	2	4	18		
C*/C	0	0	0	6	6		
C*/T	1	0	2	3	6	70%	40%
A*/C	8	2	0	8	18		
A*/A	4	0	0	0	4		
A*/G	5	1	0	0	6	39%	20%
G*/A	6	0	0	0	6		
G*/T	5	0	3	10	18		
G*/G	2	4	0	0	6	57%	44%
T*/T	0	0	0	4	4		
T*/G	0	2	5	11	18		
T*/C	0	1	0	5	6	100%	N/A

The final column analyses those mismatch bases without T bases on one or other or both sides.
*The mismatch base on the fluorescent labelled strand is marked by an asterisk.

biotin/streptavidin chemical cleavage was applied for the analysis of these mismatched bases. One strand of each heteroduplex was fluorescently labelled while the other was biotinylated and bound to streptavidin-coated magnetic beads. This methodology of chemical cleavage allowed for the analysis of two fluorescently labelled strands representing two mismatched bases per experiment. The experiment was repeated by alternately labelling the strands, forming heteroduplexes and performing chemical cleavage with $KMnO_4$ in TEAC to obtain complete analysis of all four strands (Table 1). The data are further summarised according to mismatched adenine, thymine, guanine or cytosine bases in Table 2.

Mismatched thymine bases

All 28 mismatched thymine bases were reactive and cleaved. Of these, three produced a cleavage peak of low reactivity, five were of medium reactivity and 20 were of high reactivity. Figure 1 illustrates examples of low, medium and high reactivity.

All of the reactive 28 mismatched thymine bases were analysed in Tables 1 and 2. The three weakly reactive mismatches included two T/G and one T/C. It is interesting that the two weakly reactive T/G mismatches (–82G and –4T) have the same two matched base pairs represented by the sequence CT on the 5′ side adjacent to the mismatched thymidine. Stacking or other effects may induce this low reactivity. However, a T/G mismatch of high reactivity (–54A) contains the CT matched base pair sequence on both sides of the T/G mismatch. A T/G bond is stable and reactivity may be influenced by their surrounding sequence (8).

The mode of action of TEAC is unclear. What is known is that the presence of tetraalkyl ammonium ions leads to an overall destabilisation of the duplex as the T_m is decreased (9). However, they display specific interactions with A/T base pairs and reduce the sequence specificity of duplex T_m (9). Previous speculations concerning TMAC (3) have suggested that the alkylammonium ion may have a reduced affinity for the T/T, T/G and T/C mispairs as opposed to T/A pairs. This would prevent $KMnO_4$ reactivity at matched thymine bases as opposed to mismatched thymine bases.

Comparison of OsO_4 and $KMnO_4$ in TEAC with thymine bases

To examine the difference in reactivity of mismatched thymine bases with $KMnO_4$/TEAC and OsO_4, some of the mismatches were reacted with both chemicals. In most cases the signal-to-noise ratio when using $KMnO_4$ was greater than with OsO_4. A T/G mismatch was strongly cleaved with $KMnO_4$ (Fig. 2B) but weakly with OsO_4 (Fig. 2A). The matched thymidine adjacent to the A/C mismatch (or the mismatched A) on the same mutant was strongly cleaved with $KMnO_4$ (Fig. 2B) but indistinguishable from background with OsO_4 (Fig. 2A).

Earlier studies with T/G mismatch reactivity with OsO_4 identified about a third which were unreactive (10). This situation does not appear to be replicated with $KMnO_4$ as all T/G pairs were reactive (though two were weak). A guanine base 5′ to the thymidine of a T/G mismatch guaranteed lack of reactivity with OsO_4 (10), but this is not the case with $KMnO_4$ as three such mismatches in this series (WT/–78A, WT/–37T and WT/–18C) were modified with $KMnO_4$ and this resulted in intermediate to high reactivity (Table 1).

Other bases

$KMnO_4$ has the ability to react with other bases as well as matched thymidine residues adjacent to mismatches. $KMnO_4$ is known to be reactive with free guanosine and cytidine and less with free adenosine (11). However, no studies have been reported with mismatched guanine, cytosine or adenine bases. An analysis was performed to evaluate the possibility that mismatched guanosine, cytidine and adenosine residues might be reactive with $KMnO_4$/TEAC either directly or apparently reactive due to nearby matched thymine bases (thymine bases on either side adjacent to the mismatch). Table 2 provides a summary of the analyses. Overall, 57% of guanine, 70% of cytosine and 39% of adenine mismatched bases were reactive. Of those that contained no nearby thymidine residues, 44% of guanosine, 40% of cytidine and 20% of adenosine mismatches were reactive. These are now described below.

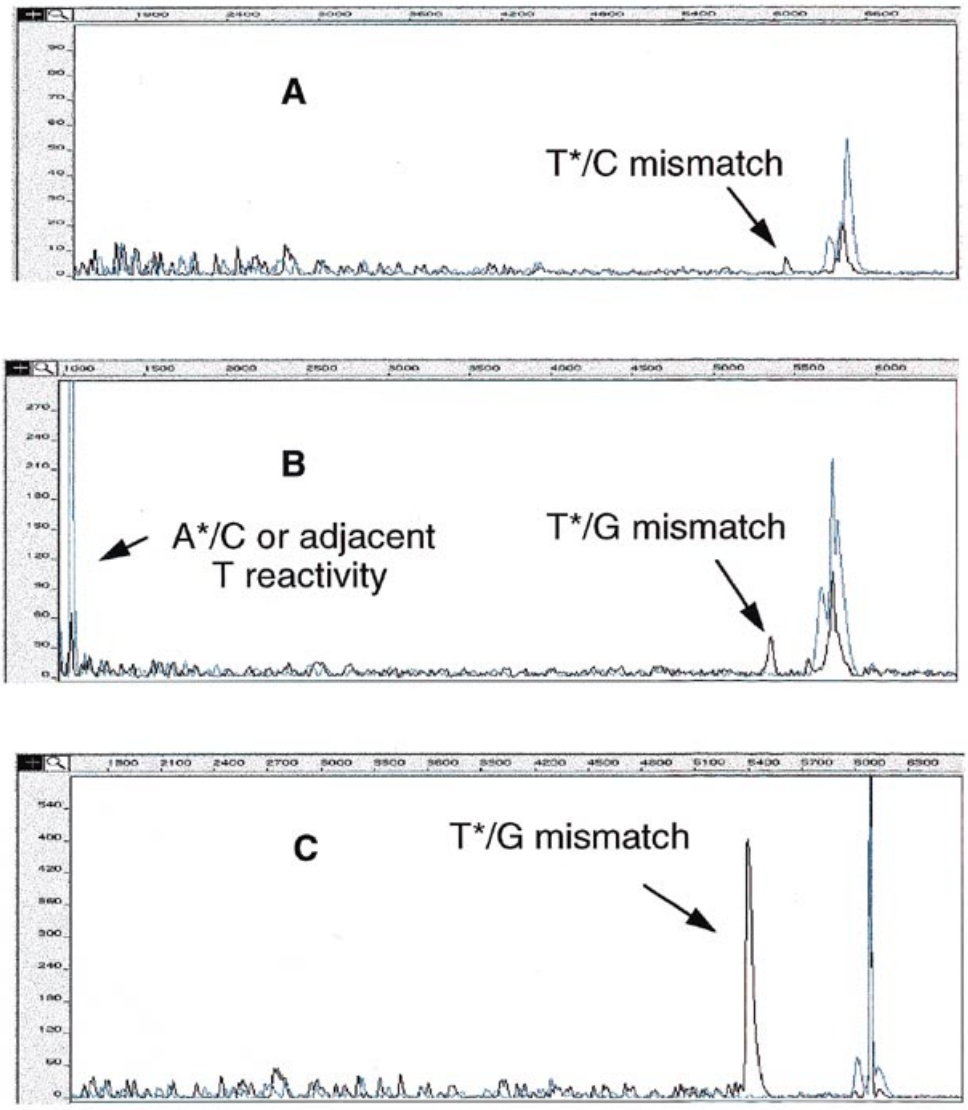


Figure 1. Traces demonstrating three different strengths of reactivity when $KMnO_4$ was incubated with heteroduplex DNA. Scans and analyses were obtained from an ABI 377 Sequencer as described in Materials and Methods. (**A**) Low reactivity shown on a T*/C mismatch (WTB/–89AF). (**B**) T*/G mismatch of medium reactivity (WTB/–85AF). (**C**) T*/G mismatch illustrating high reactivity (WTB/–54AF). The vertical scales represent fluorescence intensity. The fragments are resolved by molecular weight on the horizontal axis with increasing size from left to right. The DNA labelled with biotin is represented by B and that labelled with fluorophore designated F. The figures in the panels for the chemical cleavage reactions are colour coded. The black strand corresponds to the fluorescently labelled HEX 3′ primer strand. The blue corresponds to the 6-FAM 5′ primer strand. Adjacent T reactivity refers to the matched T adjacent to the mismatch reacting with $KMnO_4$.

Mismatched cytidine bases. Twenty-one out of 30 cytidine mismatches were reactive to varying degrees. However, some of this reactivity may have been the result of nearby matched thymine base reactivity. Table 2 indicates that five of six C/T mismatches were cleaved; four of these mismatches contained an adjacent matched thymine base. Figure 3A is an example of a C/T mismatch with no adjacent matched thymine base to the mismatch. All C/C mismatches were strongly cleaved; only half of these had an adjacent thymine base. Some C/A mismatches (10 of 18) were cleaved; seven of these contained an adjacent thymidine residue. To control for this nearby matched thymine base reactivity we analysed the apparent reactivity of mismatched cytosine bases where there were no thymine bases on either side (Table 2). The fact that there were six reactive cytidine mismatches of 15 in this category suggested that some but not all mismatched cytosine bases were indeed reactive.

Mismatched guanine bases. Thirteen out of 18 G/T mismatches were cleaved with $KMnO_4$ and TEAC. Half of these contained an adjacent thymidine. Figure 3B is an example of a G/T mismatch with no nearby thymidine residue. Most mismatched G/G bases were weakly cleaved (four of six); two of these had a nearby thymidine residue. However, one of the unreactive G/G mismatches did contain an adjacent thymidine residue. There was no evidence of reactivity in any G/A mismatches. However, only one of these G/A mismatches had an adjacent matched thymine base. When those reactive guanine mismatches without thymine bases

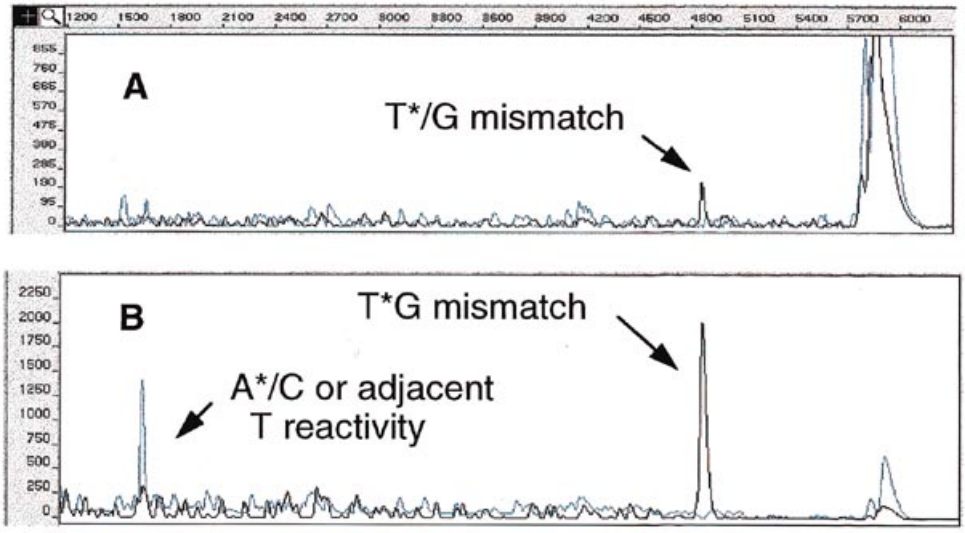


Figure 2. Comparing the reactivity of $KMnO_4$ in TEAC and OsO_4 with the same mutant WTF/–14GB in parallel conditions. (This experiment was performed in duplicate and identical results were obtained.) (**A**) OsO_4 compared to $KMnO_4$ reacts weakly with a T*/G mismatch. (**B**) $KMnO_4$ reacting strongly with the T*/G mismatch. A matched thymine base adjacent to the A*/C mismatch was also strongly cleaved.

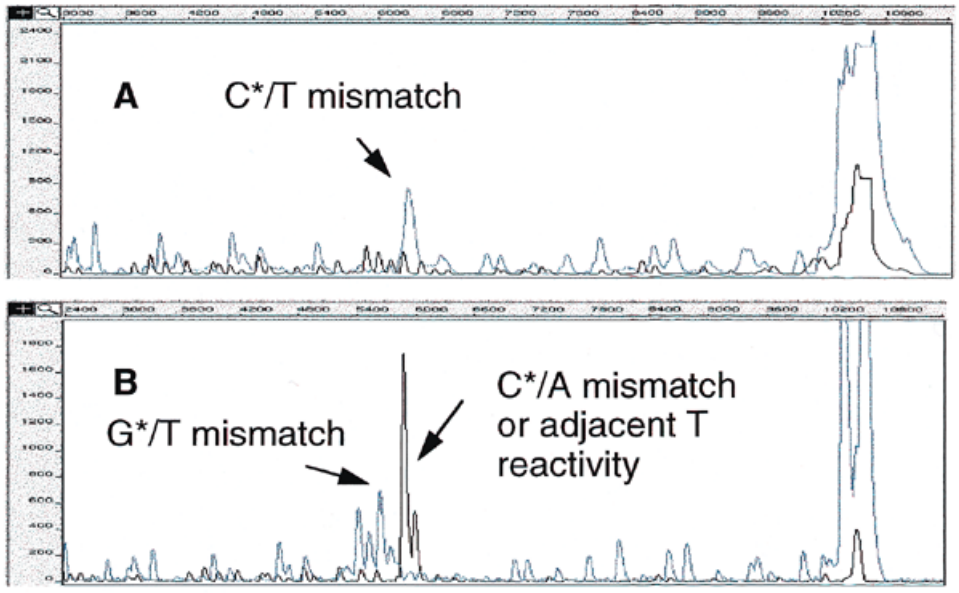
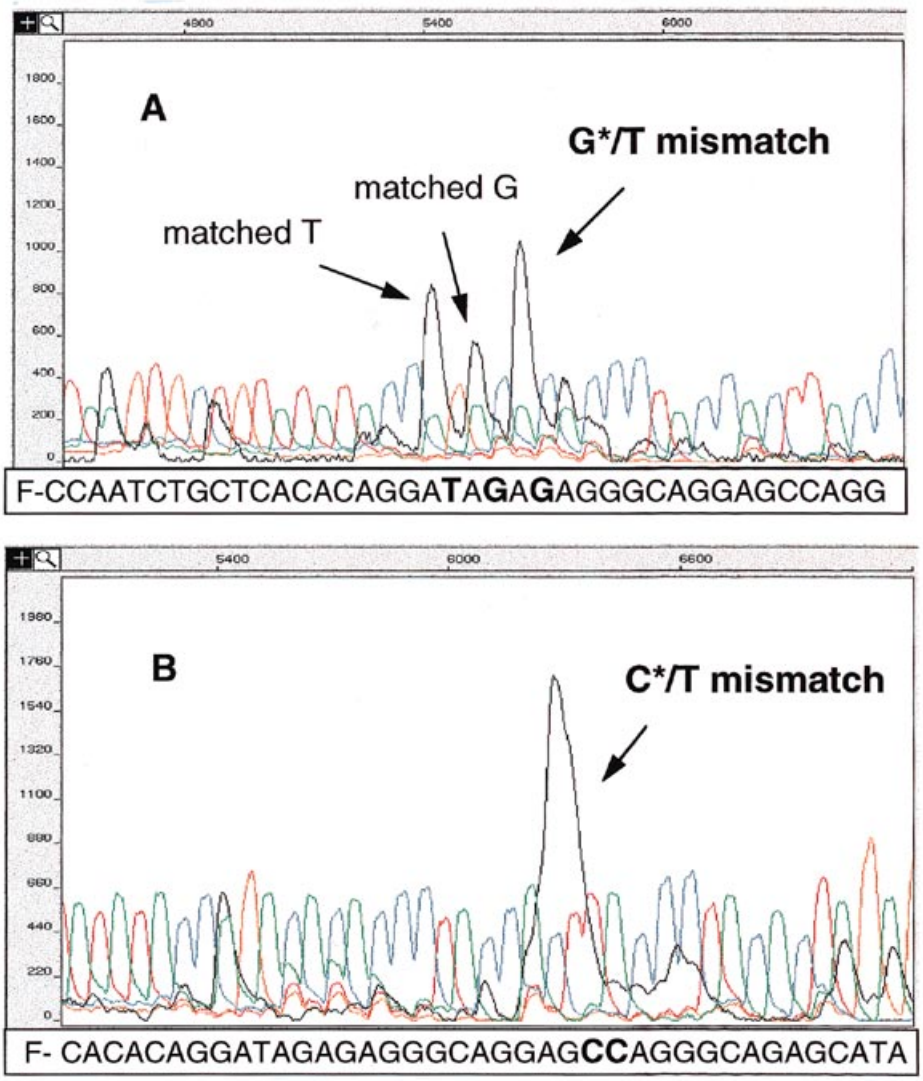


Figure 3. Reactivity of $KMnO_4$ with cytidine and guanosine mismatches. Mismatched heteroduplexes were modified with $KMnO_4$ and TEAC as described in Materials and Methods. (**A**) Reaction with a C*/T mismatch (WTF/–43AB). (**B**) Reactivity with a G*/T mismatch and a C*/A mismatch (WTF/–54AB). Adjacent T reactivity refers to the matched T adjacent to the mismatch reacting with $KMnO_4$.

on either side were analysed (Table 2) seven of 16 were reactive suggesting direct reactivity.

Mismatched adenine bases. Eleven of 28 mismatched adenine bases were reactive (three weakly). It is interesting that eight of the 18 A/C mismatches showed strong reactivity, however, six of these had a thymidine on one or other side, possibly explaining the mismatch result. When those reactive A mismatches without thymine bases on either side were analysed (Table 2), only three of 15 were reactive, suggesting that most reactive adenine mismatches are due to nearby thymine bases.

Although many guanine, cytosine and adenine mismatched bases appeared to be reactive despite no adjacent thymidine residues being present, this does not eliminate the possibility that other adjacent matched bases (G, C or A) or in fact matched bases nearby but not adjacent to the mismatch are responsible for this activity. Therefore, in some cases the exact base being modified was determined by accurately sizing the resultant cleavage



fragments by using single base resolution markers. The mismatches chosen did not have thymine bases on either side of the mismatch base on the fluorescently labelled strand. This experiment provided evidence to suggest $KMnO_4$ in TEAC reacted directly with at least some mismatched guanine bases (Fig. 4A) and cytosine bases (Fig. 4B). Other examples showed that the reactivity was sometimes due to neighbouring matched bases, most often thymine bases but also occasionally with matched guanine and cytosine bases. This was illustrated in Figure 4C where a matched thymidine adjacent to a G/G mismatch reacted with $KMnO_4$. An apparently reactive adenosine mismatch (WT/–25A) was subsequently shown to be due to reactivity of a thymine base 3 nucleotides 5′ to the mismatch (Fig. 4D). Four other nearby matched thymine bases and a guanine matched base also participated in the reactivity.

Thus, from this analysis it can be seen that mismatched guanine, cytosine and possibly adenine bases can be reactive, but are not as reactive as thymine bases (Table 2), where no unreactive examples were found throughout the series. The analysis of the former three bases is complicated by the fact that nearby reactive thymine (or other bases) may make a mismatched adenine, cytosine or guanine base appear reactive. This latter point, however, works to the advantage for the use of $KMnO_4$ for mutation detection. Thus, if we consider the 29 mutations as to whether they would be detected with $KMnO_4$ alone (Table 1), it is clear that they would be (12). This is a major feature of the use of this reagent for mutation detection. However, there was one mutation (WT/–89A, Table 1) where no reactivity was apparent on three of the strands and weak on the final one. The DNA studied here is equivalent to homozygous mutations and it is debatable whether this mutation would be detected in a heterozygous situation.

The consequences of the multiple reactivities of $KMnO_4$ with other bases under these conditions does not significantly alter the background but may alter its character. False positives in this series of results were not observed. Excessive cleavage may reduce the ability to detect multiple mismatches. Decreasing the $KMnO_4$ incubation time or decreasing the pH level of piperidine from 10 to 7–8 are two ways that may control excessive cleavage.

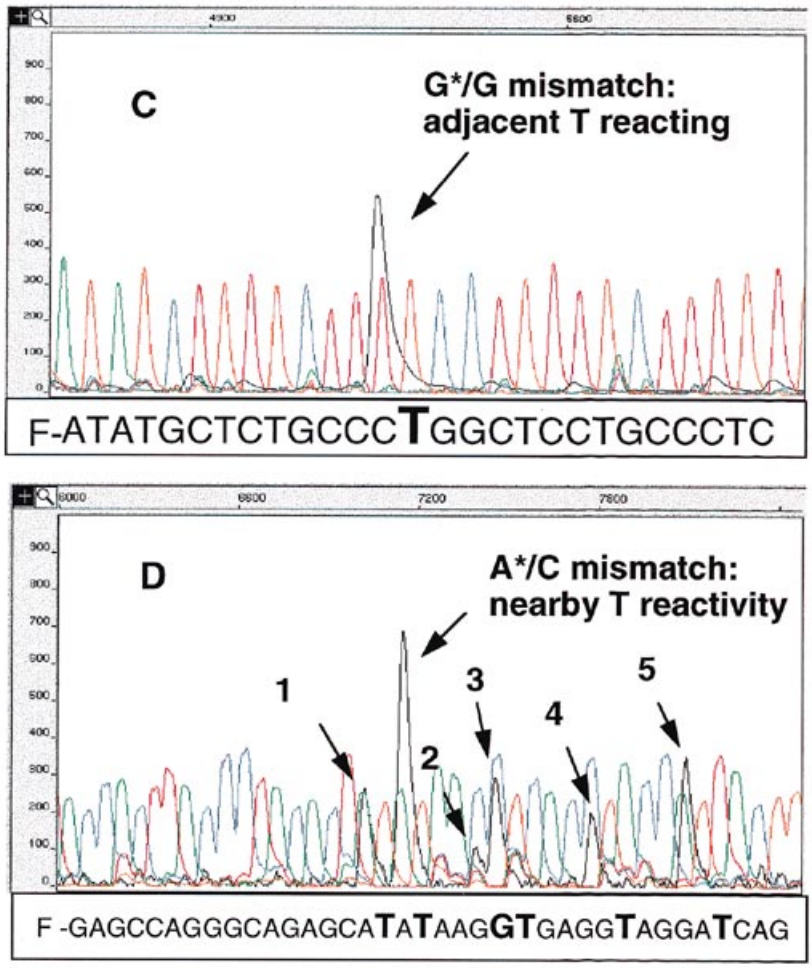


Figure 4. (Previous page and above) Gel images illustrating chemical cleavage and sequencing ladder products on an 8% polyacrylamide sequencing gel. (This experiment was performed in duplicate and identical results were obtained.) (**A**) A CCM migrates at the same position as the 70 bp sequencing ladder product indicating that bp 71, a mismatched guanosine residue of the G*/T mismatch, is modified in the chemical cleavage procedure (WTF/–54AB). $KMnO_4$ also reacts with bp 69, a matched guanosine residue, and bp 67, a thymidine residue. (**B**) CCM product migrating at the same position as the 81 and 82 bp sequencing ladder product indicating that the bp 82 and 83 cytidines of the C*/T mismatch are modified during CCM (WTF/–43AB). (**C**) CCM product migrating at the same position as the 64 bp sequencing ladder product indicating that the bp 65, a thymidine adjacent to the G*/G mismatch, is cleaved (WTF/–42GB). (**D**) CCM product migrating at the same position as the 96 bp sequencing ladder product indicating that bp 97, a thymidine residue 3 nucleotides away from the A*/C mismatch, is cleaved (WTB/–25AF). Four matched thymine base pairs at the following sequencing ladder positions (95, 102, 107 and 112) and a matched guanosine at bp 101 neighbouring the mismatch also participate in the reactivity to a minimal degree. Cleavage peak due to: **1**, a matched T base; **2**, a matched G base; **3**, a matched T base; **4**, a matched T base; **5**, a matched T base. The different colour traces illustrate the four different types of bases produced by the sequencing ladder reactions. The red trace corresponds to the cytosine bases, orange to thymine, green to adenine and blue to guanine bases. The black trace corresponds to the chemical cleavage reaction products. The sequence line of the wild-type is represented under each panel. The bases in bold react with $KMnO_4$. The letter F corresponds to the fluorescently labelled strand.

Single tube chemical cleavage of mismatch

Single tube CCM involves the combination of the two reactions to the same sample one after another (13). Normally NH_2OH is added, the DNA is ethanol precipitated and washed after incubation before the OsO_4 reaction can take place. Replacing OsO_4 with $KMnO_4$ eliminates the need for the 'clean-up' step between two reactions as the NH_2OH reaction can take place following the initial $KMnO_4$ reaction with similar reactivities. NH_2OH immediately reduces the permanganate ions hence terminating the $KMnO_4$ reaction. The excess NH_2OH added ensures that the efficiency of its modification is unchanged. Thymidine and cytidine mismatches can therefore be detected in the same tube; this gives a major benefit in time and cost. This technique was proven to work effectively in both solid (biotin-streptavidin, Fig. 5A) and liquid phase (Fig. 5B). Both internal and terminal labelling strategies are applicable to the new single tube protocol. Internal labelling is conventional and cheaper but does not provide the positional information that end labelling does. The reagents are used separately if maximal information for the detection of mismatches on both strands needs to be obtained (3).

Earlier studies using $KMnO_4$ in the toxic TMAC (3) studied six mismatches representing the three possible mismatches and the

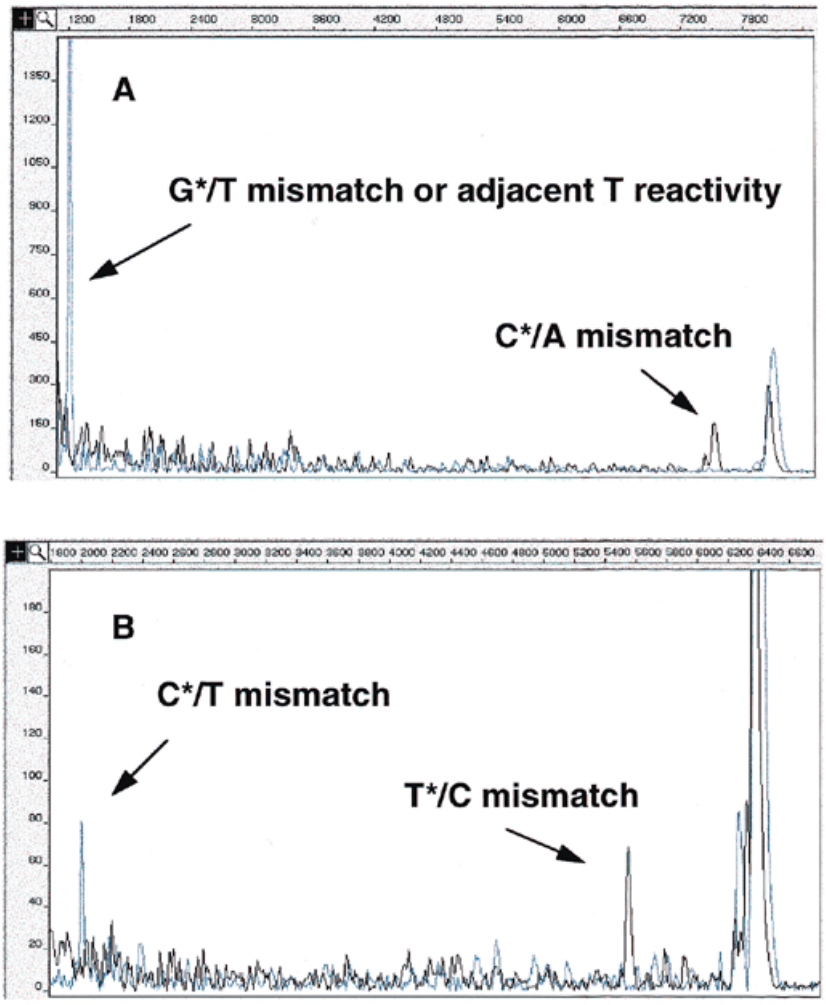


Figure 5. $KMnO_4/NH_2OH$ CCM reactions detecting both thymidine and cytidine mismatches in a single tube. This technique is demonstrated in both solid (**A**) (WTF/–85AB) and liquid phase (**B**) (WTF/–42AF) systems (Materials and Methods).

more recent $KMnO_4$/TEAC studies studied one of each thymidine mismatch type (4).

The present study was designed to study the reactivity of many examples of a range of thymine mismatches and other mismatched bases with $KMnO_4$/TEAC. This fundamental study is necessary to predict the usefulness of $KMnO_4$ and TEAC in precision mutation detection in the CCM (2).

These extensive and detailed studies report that $KMnO_4$/TEAC: (i) is less influenced by neighbouring bases than OsO_4 in its modification of thymidine mismatches (Fig. 2); (ii) is highly reactive with matched thymine bases nearby mismatched bases compared with OsO_4 (Fig. 2); (iii) to a certain extent reacts directly with guanosine and cytidine mismatches. Consequent on these findings, $KMnO_4$/TEAC would be able (based on the present series) to detect all homozygous mutations used alone without the use of NH_2OH provided all four strands are labelled. However, if only two strands were labelled this level of mutation detection would be less (93–97%). Also, for examination of heterozygous mutations, heteroplasmic mutations and mutations in a wild-type background in tumours, detection would be maximal with the combined use of $KMnO_4$/TEAC with NH_2OH.

Conventional CCM is limited by the time-consuming nature of the technique and by its toxicity which is mainly due to the use of OsO_4. TMAC, described earlier (3), is far more toxic than the recently described TEAC (4) and this may explain the lack of use of this earlier protocol. $KMnO_4$ in TEAC is safer and less toxic than OsO_4 (4). Reactivity with mismatched bases and matched bases nearby the mismatch is higher with $KMnO_4$/TEAC in contrast to OsO_4. To a certain extent $KMnO_4$ and TEAC, unlike OsO_4, react with guanosine mismatches (Fig. 4A). The cost of $KMnO_4$ and TEAC is considerably less in comparison to the cost of OsO_4. For all these reasons the replacement of OsO_4 with $KMnO_4$ and TEAC is desirable and supports earlier findings (4).

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