Research Report

Analysis of Duplex DNA by Triple Helix Formation: Application to Detection of a p53 Microdeletion

Wendy M. Olivas and L. James Maher, III
Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA

ABSTRACT

Conventional methods for mutation detection include Southern hybridization, direct sequencing of PCR products and single-strand conformation polymorphism analysis. We present an additional screening method that employs oligonucleotide-directed DNA triple helix formation to detect mutations within homopurine sequences. The proposed strategy is simple and may be of particular value when screening many DNA samples for changes involving particular homopurine sites. We have applied the method to the analysis of a clinically relevant 8-bp microdeletion in the human p53 tumor suppressor gene. Affinities of oligonucleotide probes toward radiolabeled wild-type and mutant p53 DNA duplexes were quantitated by electrophoretic mobility shift assays. Recombinant plasmids carrying wild-type or microdeleted forms of the p53 homopurine sites of interest were created. Dimethyl sulfate footprinting was used to verify intended probe specificities. Duplex PCR products amplified from plasmid constructs were directly probed by incubation with labeled oligonucleotides. After electrophoresis and autoradiography, patterns of triple helix formation allowed discrimination between the mutant and wild-type p53 sequences. Direct DNA analysis by triple helix formation may simplify other procedures that normally require DNA denaturation and hybridization.

INTRODUCTION

Several methods for detecting specific mutations in clinical DNA samples are currently available, including Southern blot analysis, restriction fragment length polymorphisms (RFLPs), direct sequencing of polymerase chain reaction (PCR) products and single-strand conformation polymorphism (SSCP) analysis (13). Each method has certain advantages and disadvantages. In the present study, we show that oligonucleotide binding to duplex DNA (triple helix formation) provides an additional, simple approach to detection of certain mutations within homopurine sequences.

Oligonucleotide-directed triple helix formation permits sequence-specific recognition of purine sequences in duplex DNA by discrimination between hydrogen bonding patterns available in the DNA major groove (4,5,9,12). Oligonucleotide binding results from the formation of contiguous base triplets in either of two patterns, termed the "pyrimidine motif" and the "purine motif" (9). Previous studies have suggested the potential value of oligonucleotide-directed triple helix formation for a number of applications, including site-specific cleavage of duplex DNA (12), sequence-specific affinity-capture of DNA fragments (6) and promoter-specific gene repression (5,9).

The oligonucleotide probes used in the present study bind duplex DNA by means of the pyrimidine motif under acidic conditions that stabilize protonated C+GC base triplets (Figure 1). Single triplet mismatches between an oligonucleotide probe and DNA duplex have previously been shown to destabilize triple helix formation in this motif under appropriate stringent conditions (12). We therefore hypothesized that triple helix formation might provide a sensitive method to detect mutations in homopurine DNA sequences by monitoring changes in hydrogen bond contacts in the DNA major groove.

To investigate this possibility, we examined the human p53 tumor suppressor gene for homopurine sequences that overlap documented mutations of clinical interest. The 1760-bp p53 cDNA contains seven homopurine sequences that are at least 10 bp in length. One of these sequences contains the site of a documented 8-bp microdeletion (3). We present a simple method in which triple helix formation successfully differentiated PCR products derived from wild-type and microdeleted forms of the p53 sequence. The method is rapid and inexpensive and consists of four steps: (i) PCR amplification of the target sequence, (ii) a binding reaction involving labeled oligonucleotide probes, (iii) electrophoresis under acidic conditions and (iv) autoradiography. This method might complement other assays used to screen multiple DNA samples for particular mutations of interest.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I Klenow fragment and Vent™ DNA polymerase were obtained from New England Biolabs (Beverly, MA, USA). Protected 5-methylcytosine phosphoramidite was

obtained from Glen Research (Sterling, VA, USA). [γ-^{32}P]ATP and [α-^{32}P]dATP were obtained from Amersham (Arlington Heights, IL, USA).

Oligonucleotides

Synthetic oligonucleotides were purified and quantitated as described (11). Oligonucleotides were radiolabeled using polynucleotide kinase and [γ-^{32}P]ATP, followed by ethanol precipitation. Oligonucleotides for target duplexes were annealed and purified using QIAEX™ particles (Qiagen, Chatsworth, CA, USA).

Electrophoretic Mobility Shift Assays

Binding reactions contained labeled duplex (50000 cpm; ca. 0.4 pmol), 1 µl of 10× binding buffer (1 M sodium acetate [NaOAc] pH 5.0, 50 mM NaCl, 100 mM $MgCl_2$), 1 µl tRNA (1 mg/ml), 1 µl probe oligonucleotide (to yield the indicated final concentration) and H_2O in a final volume of 10 µl. Reactions were incubated at 22°C for at least 5 h. Reactions were supplemented with 1 µl of an 80% glycerol solution containing bromophenol blue and were loaded on a 20% native polyacrylamide gel (19:1 acrylamide:bisacrylamide) prepared in 0.1 M NaOAc pH 5.0, 1 mM $MgCl_2$. Electrophoresis was performed in this buffer (with recirculation) at 4°C overnight (9 V/cm). The resulting gel was autoradiographed and quantified by scintillation counting of excised gel bands.

Analysis of Gel Mobility Shift Assays

The apparent fraction of target duplex bound by probe oligonucleotide, Q, was calculated for each concentration of probe oligonucleotide using the definition:

$$Q = cpm_{triplex} / (cpm_{triplex} + cpm_{duplex})$$

where $cpm_{triplex}$ and cpm_{duplex} represent radioactive counts per minute for triple-helical and duplex complexes, respectively. To correct for background effects, the fraction of duplex in triple-helical form was then converted to a scaled score, F:

$$F = (Q - Q_{low}) / (Q_{high} - Q_{low})$$

where Q_{low} and Q_{high} correspond to Q values obtained in the presence of 0 or 2.5 µM oligonucleotide probe, respectively. This treatment assumes that the fraction of triple helix in the absence of oligonucleotide probe must be 0, and that the fraction of triple helix approaches 1.0 in binding reaction containing 2.5 µM specific oligonucleotide probe. These assumptions are valid for the experiments described here. Values of the apparent dissociation constant, K_d, were obtained by least squares fitting of the data to the binding isotherm:

$$F = [O] / (K_d + [O])$$

where $[O]$ is the oligonucleotide probe concentration.

Dimethyl Sulfate Footprinting

Wild-type and deletion model duplex oligonucleotides were ligated into plasmid pG_5E4T that had been cleaved by *Hin*dIII and *Pst*I (10). Identities of the resulting clones were confirmed by sequencing. A 2704-bp *Bam*HI-*Kpn*I restriction fragment was then prepared and uniquely end-labeled by using Klenow fragment of DNA polymerase I. Labeled fragment (50000 cpm; ca. 0.4 pmol) was incubated with 1 µl of 10× binding buffer (1 M NaOAc pH 5.0, 50 mM NaCl, 100 mM $MgCl_2$), 1 µl sheared calf thymus DNA (2.5 mg/ml), 1 µl oligonucleotide probe (10 µM), and H_2O in a total volume of 10 µl. Binding reactions were incubated overnight at 22°C. Dimethyl sulfate (DMS) [1 µl of a 4% (vol/vol) aqueous solution] was added to each reaction and allowed to incubate for 8 min at 4°C. Reactions were precipitated with ethanol, treated with hot piperidine, and electrophoresed on a 20% polyacrylamide sequencing gel (19:1 acrylamide:bisacrylamide) containing 7.5 M urea in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) buffer and autoradiographed.

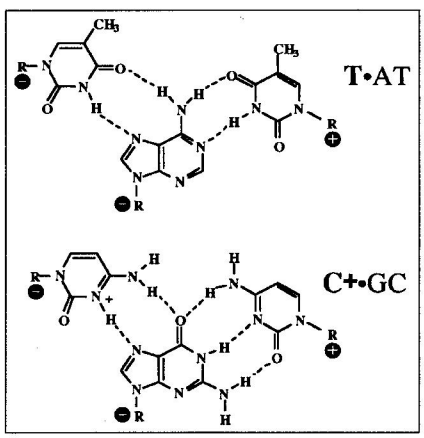

Figure 1. DNA triple helix formation in the pyrimidine motif. T·AT and C$^+$·GC base triplets are involved in duplex DNA recognition by oligonucleotide probes. Filled circles indicate strand polarities.

Probing PCR Products by Triple Helix Formation

A 496-bp DNA region containing the p53 duplex insert was amplified by PCR from the plasmids described above using Vent DNA polymerase. Oligonucleotide binding reactions containing 2 µl of the resulting PCR-amplified DNA, 1 µl labeled oligonucleotide probe, 1 µl unlabeled oligonucleotide probe (5 µM), 2 µl of 10× binding buffer (1 M NaOAc pH 5.0, 50 mM NaCl, 100 mM $MgCl_2$), 2 µl tRNA (1 mg/ml) and 10 µl H_2O were incubated at 22°C for 1 h. Binding reactions were then supplemented with 1 µl of 80% glycerol loading buffer and electrophoresed on a 5% native polyacrylamide gel (19:1 acrylamide:bisacrylamide) at 4°C in 1× running buffer (0.5 M NaOAc pH 5.0, 1 mM $MgCl_2$) with recirculation (27 V/cm), followed by autoradiography.

RESULTS

Experimental Design

A documented p53 mutation in a hepatocellular carcinoma patient consists of an 8-bp deletion starting at the third position of codon 285 within exon eight of the p53 gene (3). The deletion disrupts a 12-bp homopurine/homopyrimidine sequence (denoted as Area I in Figure 2). A nearby 12-bp homopurine/homopyrimidine sequence remains intact (denoted as Area II in Figure 2). We wished to determine if triple helix formation with probes to Areas I and II could discriminate mutant and wild-type forms of the p53 gene. To study this possibility, we first designed synthetic duplexes containing 38-bp sequences corresponding to wild-type or microdeletion mutant forms of the p53 gene. Oligonucleotide Probes I and II were synthesized so as to specifically bind wild-type homopurine Areas I and

II, respectively, by means of the pyrimidine triple helix motif. Because Area I is disrupted by the 8-bp microdeletion, Probe I is predicted to form only 7 of 12 possible base triplets with the mutant p53 duplex, relative to the wild-type duplex. Thus, binding of Probe I might be used to differentiate between wild-type and mutant p53 sequences, with Probe II providing a positive control. Three kinds of experiments were performed to determine whether differential third-strand binding could discriminate between mutant and wild-type p53 sequences: (i) electrophoretic mobility shift assays allowed measurement of probe affinities, (ii) dimethyl sulfate footprinting assays confirmed probe specificities and (iii) wild-type and mutant sequences (amplified by PCR) were distinguished by differential binding of labeled probes.

Affinities of Oligonucleotide Probes

Electrophoretic mobility shift assays have been used qualitatively to detect the formation of triple-helical DNA complexes, and quantitatively to measure oligonucleotide affinities for duplex DNA (4). In experiments to study the wild-type target duplex (Figure 3A), we observed that increasing concentrations of both Probe I and Probe II resulted in gel shifts representing triple helix formation at pH 5.0 (Figure 3A, compare lanes 1–9 and 10–18). Measured dissociation constants (K_d ± standard error of the mean) for Probes I and II were $(8.1 \pm 0.7) \times 10^{-8}$ M and $(2.36 \pm 0.18) \times 10^{-7}$ M, respectively. Thus, although there is a threefold difference in the K_d values for Probes I and II, both are in the sub-micromolar range.

In contrast to these results, Probe I did not detectably bind the mutant target duplex, even at concentrations up to 2.5 μM (Figure 3B, lanes 1–9), whereas the binding of Probe II was not disrupted in the mutant duplex (Figure 3B, lanes 10–18). The measured dissociation constant for the latter interaction was $(9.4 \pm 1.8) \times 10^{-8}$ M. The approximately twofold difference in binding affinities of Probe II for wild-type and mutant targets was unexpected and may reflect a slight alteration in the structure of Area II due to changes in the Area I sequence. Taken together, these data indicate that Probe II binds both wild-type and mutant targets with relatively high affinity, while Probe I binds only the wild-type target under the conditions tested.

Confirmation of Oligonucleotide Specificity

DMS footprinting assays were performed to confirm that Probes I and II exhibit the intended specificities when binding to their target sites (Figure 4). Protection of guanine N7 from DMS modification is conferred by triple helix formation (1). When cloned into plasmids, the wild-type target duplex shows protection of all guanines in Area I by Probe I and all but the 3′ guanine of Area II by Probe II (compare Figure 4, lanes 3 and 4). Lack of protection at the 3′ guanine of Area II may be due to the synthetic constraint that the 3′ cytosine of Probe II is not methylated, a modification that enhances triple helix formation (14).

In contrast, the mutant target duplex shows no guanine protection by Probe I, while Area II is fully protected by Probe II (Figure 4, compare lanes 7 and 8). Taken together, these DMS footprinting data confirm the intended binding specificities of the oligonucleotide probes.

Discrimination Between Wild-Type and Mutant Forms of the p53 Sequence after PCR Amplification

We wished to determine if mutation detection might be performed by coupling PCR and triple helix formation. We therefore used PCR to amplify 496-bp DNA molecules from regions of plasmid DNA containing the wild-type and mutant p53 sequences of interest. Samples of the resulting unpurified PCR products were directly mixed with labeled probe oligonucleotides in pH 5.0 buffer and electrophoresed in a 5% native polyacrylamide gel at pH 5.0 in the presence of 1 mM $MgCl_2$. The resulting gel was autoradiographed to assess probe binding (Figure 5). Labeled Probes I and II both bound the 496-bp PCR product generated from the wild-type p53 sequence (Figure 5, lanes 4 and 5). In contrast, Probe I did not detectably bind the 496-bp PCR product generated from the mutant p53 sequence, while binding of Probe II was

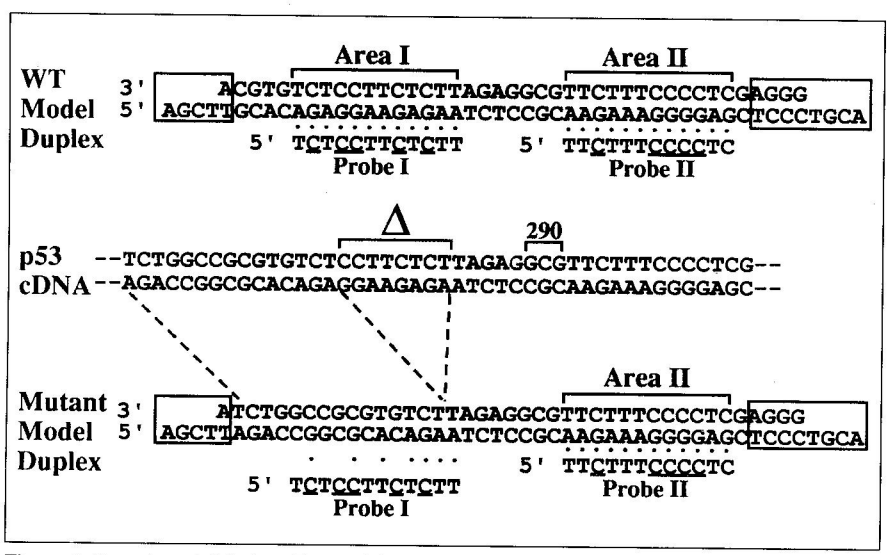

Figure 2. Experimental design. The partial nucleotide sequence of wild-type p53 cDNA is shown in the center. The position of codon 290 is shown for reference. The position of a clinically relevant 8-bp microdeletion is indicated (Δ). The nucleotide sequence of a synthetic oligonucleotide duplex model of the wild-type region is shown above. Area I and Area II indicate homopurine/homopyrimidine sequences. Probe I and Probe II indicate sequences used for oligonucleotide-directed DNA triple helix formation, with the underlined C residues representing 5-methylcytosine. Dots between probes and duplex targets represent proper base triplets. Boxed regions represent added sequences containing *Hin*dIII and *Pst*I restriction endonuclease recognition sites. The nucleotide sequence of a synthetic oligonucleotide duplex model of the microdeleted p53 allele is shown below. Dashed lines emphasize the frameshift in the mutant model sequence due to the 8-bp deletion. This mutation disrupts the sequence of Area I without affecting Area II.

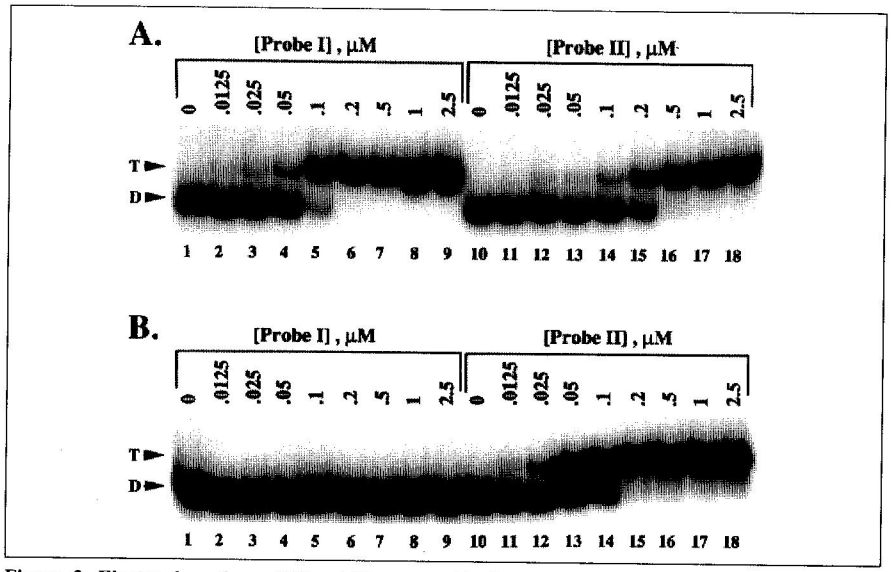

Figure 3. Electrophoretic mobility shift assays. (A) Increasing concentrations of oligonucleotide Probes I (lanes 1–9) and II (lanes 10–18) were added to labeled wild-type target duplex. Arrowheads represent the location of the free duplex (D) and triple-helical complex (T). (B) Increasing concentrations of oligonucleotide Probes I (lanes 1–9) and II (lanes 10–18) were added to labeled mutant target duplex. Arrowheads represent the location of the free duplex (D) and triple-helical complex (T).

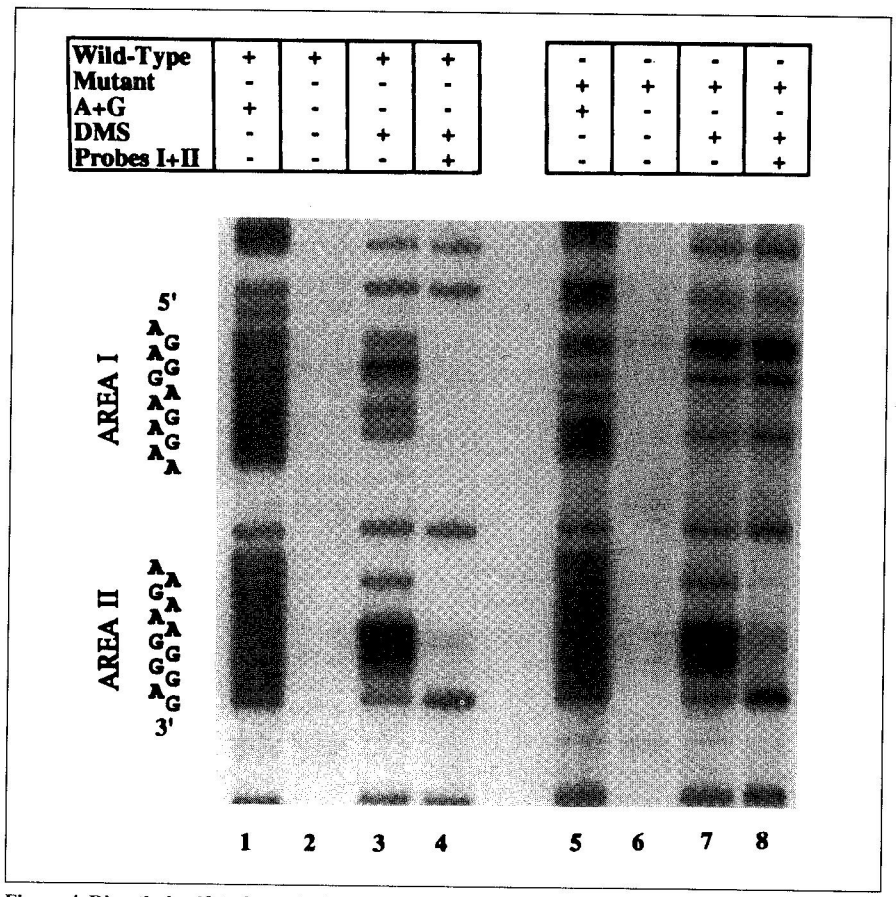

Figure 4. Dimethyl sulfate footprinting assay. Wild-type (lanes 1–4) and mutant (lanes 5–8) target duplexes were sub-cloned into plasmids and subjected to DMS modification in the absence or presence of oligonucleotide Probes I and II (lanes 3–4 for wild-type, lanes 7–8 for mutant). Lanes 1 and 5 contain A + G ladders of the target sites, while lanes 2 and 6 show background levels of DNA cleavage without DMS treatment. Sequences of homopurine Areas I and II are indicated.

still evident (Figure 5, lanes 2 and 3). We conclude that an assay consisting only of PCR amplification, probe binding, electrophoresis and autoradiography can permit discrimination between the wild-type and microdeleted p53 sequences in question.

DISCUSSION

We have presented a simple method for duplex DNA analysis based on triple helix formation. The method exploits the ability of certain oligonucleotides to monitor DNA sequences in the major groove without requiring denaturation of the double-helical DNA target and might be directly applied for general screening of mutations affecting homopurine sequences. It should be emphasized that the described procedure for oligonucleotide probing of PCR products involves unusual binding and electrophoresis conditions. For triple helix formation in the pyrimidine motif, it is critical that binding reactions and subsequent electrophoresis be performed in the presence of Mg^{++} ions under acidic conditions (pH 5.0).

Because the triple helix DNA analysis method does not require DNA denaturation, it is relatively rapid and might facilitate certain DNA screening procedures. For example, when a particular homopurine sequence is being screened for mutations, PCR products might initially be analyzed by SSCP to determine which DNA samples contain detectable mutations. The addition of labeled oligonucleotide probes to PCR products from apparent mutants would immediately determine whether any mutations found in the first step affect the homopurine sequence of interest. Finally, direct sequencing of mutations confirmed by the triple helix analysis could precisely identify the lesions.

Application of the current triple helix analysis method is obviously limited to detection of lesions that disrupt homopurine sequences on the order of 10 consecutive bp. The significance of this limitation should be judged in light of two additional considerations. First, homopurine sequences of 10 or more consecutive bp are statistically overrepresented in mammalian genomes, occurring approximately once every 250 bp by some estimates (2). In fact, this is precisely the frequency at which such sequences occur in the p53 cDNA. Therefore, it may be possible to identify many mutations of clinical significance that affect such sequences. Second, future methods may relax the requirement for continuous homopurine target sequences (1,7,8). Such improvements may extend the applicability of DNA analysis by triple helix formation to a larger number of possible sequences.

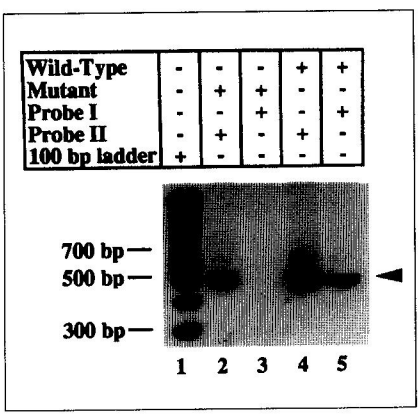

Figure 5. Discrimination between wild-type and mutant PCR products. 496-bp DNA fragments (arrowhead) containing mutant (lanes 2–3) or wild-type (lanes 4–5) p53 target sequences were amplified by PCRs. Samples of the PCRs were directly mixed with labeled Probes I or II, electrophoresed as described in Materials and Methods and autoradiographed. Lane 1 contains a 100-bp ladder.

ACKNOWLEDGMENTS

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Address correspondence to:

L. James Maher, III
Eppley Institute for Research in Cancer and Allied Diseases
University of Nebraska Medical Center
600 S. 42nd Street
Omaha, NE 68198-6805, USA
bitnet: jmaher@molecular.unmc.edu