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Linked oligodeoxynucleotides show binding cooperativity and can selectively impair replication of deleted mitochondrial DNA templates

Robert W. Taylor, Theresa M. Wardell, Bernard A. Connolly[1], Douglass M. Turnbull and Robert N. Lightowlers*

Department of Neurology and [1]Department of Biochemistry and Genetics, The Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, UK

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ABSTRACT

Mutations in mitochondrial DNA (mtDNA) cause a spectrum of human pathologies, which predominantly affect skeletal muscle and the central nervous system. In patients, mutated and wild-type mtDNAs often co-exist in the same cell (mtDNA heteroplasmy). In the absence of pharmacological therapy, a genetic strategy for treatment has been proposed whereby replication of mutated mtDNA is inhibited by selective hybridisation of a nucleic acid derivative to the single-stranded replication intermediate, allowing propagation of the wild-type genome and correction of the associated respiratory chain defect. Previous studies have shown the efficacy of this anti-genomic approach *in vitro*, targeting pathogenic mtDNA templates with only a single point mutation. Pathogenic molecules harbouring deletions, however, present a more difficult problem. Deletions often occur at the site of two short repeat sequences (4–13 residues), only one of which is retained in the deleted molecule. With the more common larger repeats it is therefore difficult to design an anti-genomic molecule that will bind selectively across the breakpoint of the deleted mtDNA. To address this problem, we have used linker-substituted oligodeoxynucleotides to bridge the repeated residues. We show that molecules can be designed to bind more tightly to the deleted as compared to the wild-type mtDNA template, consistent with the nucleotide sequence on either side of the linker co-operating to increase binding affinity. Furthermore, these bridging molecules are capable of sequence-dependent partial inhibition of replication *in vitro*.

INTRODUCTION

Human cells contain an extranuclear, autonomously replicating genome, mtDNA. Housed in the mitochondrial matrix, this circular duplex of 16 569 bp encodes 13 polypeptides, all of which are essential components of the mitochondrial respiratory chain. In addition to these mitochondrial (mt) mRNAs, the genome encodes 22 mt tRNAs and 2 mt rRNAs, the complete set required for intramitochondrial protein synthesis. Defects in this genome are an important cause of progressive muscle and neurological diseases, which often result in severe disability and premature death (1,2). In a recent population study in the North East of England the minimum incidence of pathogenic mtDNA mutations was calculated at ~1:8000 individuals (3). The genetic lesion may involve either a mtDNA rearrangement (deletion or duplication) or a point mutation, resulting in the impairment of cellular oxidative phosphorylation. Patients often harbour mutated and wild-type mtDNA molecules within the same cell and tissue, a phenomenon known as mtDNA heteroplasmy. The percentage of wild-type mtDNA is possibly the critical determinant of expression of the genetic defect and, hence, the clinical phenotype. Although this threshold level is dependent upon both the nature of the mutation and the tissue affected, many pathological mtDNA mutations are highly recessive, with biochemical dysfunction only becoming apparent with low levels of wild-type mtDNA (4,5). Despite widespread recognition of their clinical importance and considerable progress in developing techniques to define causative genetic mutations, there remains no effective treatment for these disorders (6).

On account of the heteroplasmic and recessive nature of many pathogenic mtDNA mutations, we have proposed a strategy for therapy which takes advantage of the relatively long period over which mtDNA is single stranded during replication: inhibition of mutated mtDNA replication by selective binding of anti-genomic nucleic acid derivatives. By allowing selective replication of the wild-type genome, the relative amount of wild-type mtDNA would increase, correcting the biochemical lesion and preventing disease progression (6,7). Our initial experiments pursued this strategy *in vitro*. We demonstrated that an 11mer sequence-specific peptide nucleic acid (PNA) complementary to a region of human mtDNA sequence containing the pathogenic A8344G MERRF point mutation (PNA-MERRF) was able to selectively bind and inhibit run-off replication of a single-stranded template by

*To whom correspondence should be addressed. Tel: +44 191 222 8028; Fax: +44 191 222 8553; Email: r.n.lightowlers@ncl.ac.uk

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

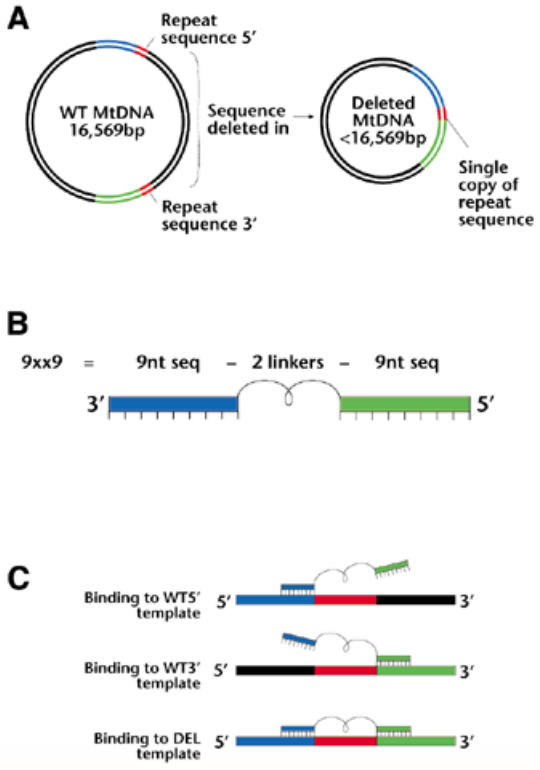

Figure 1. Schematic representation to show how a bridging oligonucleotide can be used to target deleted mtDNA molecules. (**A**) A series of mainly muscle and neurodegenerative disorders are caused by deletions in the mitochondrial genome. The mitochondrial genome contains many short repeated sequences, which are often the site at which deletions occur. Possibly due to slippage during replication, the resolved molecule is deleted for a defined region of mtDNA, but crucially retains one copy of the repeat sequence. (**B**) Representation of the bridging oligonucleotides used in this study. The bridging oligomers each contain two 9mers of complementarity to nine residues immediately proximal and distal to the duplicated sequence present in the deleted templates. The two 9mers are joined together via two polyoxyethylene linker molecules. (**C**) The bridging oligomers have nine residues of complementarity to only the distal or proximal side of the deletion breakpoint in the wild-type templates (WT5′ or WT3′) but both 9mer sequences bind to the deleted template, potentially increasing the binding affinity of the bridging oligomers for the deleted templates. Consequently, these bridging oligomers will selectively target the deletion breakpoint in the single-stranded mtDNA replication intermediate of the deleted molecule, inhibiting replication.

DNA polymerase γ (8). Identical concentrations of this PNA did not inhibit the replication of a wild-type template that differed by just a single substitution. Furthermore, by conjugating PNA-MERRF to a mitochondrial pre-protein or to a lipophilic cation, we have been able to target this anti-genomic molecule to mitochondria in human cultured cells (9,10). Although this approach has great potential for targeting pathogenic molecules that differ by only a single base, we now wish to adapt this anti-genomic approach to inhibit replication of pathogenic mtDNA carrying large deletions.

Large-scale mtDNA rearrangements represent some 60% of reported mtDNA gene defects (1). Mitochondrial genomes harbouring deletions have been observed at high levels in patients with progressive external ophthalmoplegia, Kearns–Sayre syndrome (KSS) and Pearson marrow/pancreas syndrome (11,12) and may accumulate during normal human ageing (13). Deletions typically occur at the site of repeat sequences of 4–13 bp, only one of which is lost in the deleted molecule (2,14,15). As such, the repeat sequence and either the 3′ or 5′ flanking sequence is common to both the wild-type and deleted molecule (Fig. 1). Thus, dependent on the size of the repeat sequence, the deleted genome is refractory to our standard anti-genomic approach as there is no unique sequence of suitable length that can be targeted to give selective hybridisation at physiological temperatures. However, if the repeat sequence could be bridged, the unique proximity of the 3′ and 5′ flanking regions in the deleted genome could be exploited for selective hybridisation (Fig. 1C).

In an attempt to bridge the repeat sequence we have synthesised anti-genomic ODNs containing flexible linker moieties and investigated their binding properties using thermal melt spectrophotometry and surface plasmon resonance techniques. Our results demonstrate that the 5′ and 3′ regions of linker ODNs were able to act cooperatively in increasing the binding affinity of the molecule to templates mimicking known mtDNA deletion sequences, as compared to either the 3′ or 5′ regions alone. In bridging the repeat sequence these molecules were also able to partially inhibit replication from single-stranded human mtDNA templates containing deletion breakpoints, thus confirming the potential of our anti-genomic strategy for treating patients with deletions of the mitochondrial genome.

MATERIALS AND METHODS

Oligodeoxynucleotide design and synthesis

The studies undertaken in this paper were based upon a 6930 bp deletion (nucleotides 7502–7506/14428–14432 of the Cambridge reference sequence; 16) involving a 5 bp (5′-CCATG-3′) direct repeat, originally described in a patient with KSS (patient K7 in 15). Studies were also performed on the breakpoint sequence of the mtDNA ‘common’ deletion (also known as the 4977 bp deletion), nucleotides 8470–8482/13447–13459, which occurs at a 13 bp repeat sequence (14). All anti-genomic ODNs were 3′-biotin-labelled to prevent initiation of replication during the *in vitro* run-off assay and to facilitate immobilisation on streptavidin-coated sensor chips for kinetic analysis.

The Spacer 18 phosphoramidite linker (Glen Research) is a polyoxyethylene spacer modifier that can be used to introduce a linker into a synthetic nucleic acid sequence. These spacer molecules were incorporated into bridging ODNs to span the deletion breakpoint repeat sequences.

For the bridging ODN targeted to templates containing the 5 nt repeat sequence (9XX9^{DEL5}, Table 1) the flanking nucleotides were complementary to the nine residues immediately 5′ and 3′ to the repeat in template DEL5 (nucleotides 14441–14433 and 7501–7493, inclusive). As it is not necessary to bridge the entire sequence, an ODN was designed to bridge only 9 of the 13 nt repeat retained in mtDNA with the common deletion (9XX9^{DEL13}, Table 1). Consequently, the 9 nt flanking the linkers was identical to residues 8463–8471 and 13458–13466, inclusive. A comprehensive list of all the anti-genomic ODNs and templates used in these studies is given in Tables 1 and 2, respectively. All synthetic ODNs were synthesised and HPSF purified by MWG-Biotech UK Ltd.

Table 1. Sequences of biotinylated inhibitory anti-genomic oligomers used in this study

ODN designation[a]	Sequence (5′→3′)	Base pair complementarity to		
		Deleted template	5′ WT template	3′ WT template
$9XX9^{DEL5}$	ccatggcct XX cctcaggat	18	9	9
$9\text{-}5\text{-}9^{DEL5}$	ccatggcct CCATG cctcaggat	23	14	14
$3'\text{-}9mer^{DEL5}$	ccatggcct	9	0	9
$5'\text{-}9mer^{DEL5}$	cctcaggat	9	9	0
$9XX9^{DEL13}$	accacctac XX cattggcag	18	9	9
$9\text{-}9\text{-}9^{DEL13}$	accacctac CTCCCTCAC cattggcag	27	18	18

[a]Linker molecules are designated X. Sequence denoted in upper case corresponds to the duplicated sequence at the deletion breakpoint.

Surface plasmon resonance techniques for determining binding kinetics

A BIAcore 2000 instrument was used in all binding experiments in conjunction with SA5 (research grade) sensor chips that had been pre-coated with dextran–streptavidin. Unless otherwise stated, all experiments were performed at 25°C using 1× HBS running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.05% v/v Surfactant P-20).

Immobilisation of biotinylated anti-genomic ODNs

The microfluidic system of the instrument consists of four detection surfaces located in four independent flow cells, which may be accessed individually (e.g. for ligand capture) or in multi-channel mode as used in binding studies. For each sensor chip flow cell 1 was left empty and served as a control for non-specific binding of analyte to the chip surface. Prior to immobilisation of biotinylated ODNs the sensor chip was washed three times with 1 M NaCl, 50 mM NaOH in 1× HBS and a further three times with 50 mM NaOH in 1× HBS to precondition the surface. Biotinylated ODNs (400 nM) were manually injected using a flow rate of 10 µl min^{-1}. Following three injections of the regenerating solution (50 mM NaOH), ODN equivalent to 650–700 resonance units were immobilised. Permutations of anti-genomic ODNs were immobilised on six separate sensor chips (exact permutations available on request).

Kinetic analysis of anti-genomic ODNs hybridising to mtDNA templates

The effect of different flow rates was investigated to determine whether mass transfer of analyte to ligand could in any way influence the kinetic measurements. No appreciable differences in rate constants were found with flow rates of 10–30 µl min^{-1}. The binding of mtDNA analytes to the immobilised ODNs was therefore assessed using a flow rate of 20 µl min^{-1}. Experimental working stocks of analyte templates (20 nM–5 µM) were made prior to each experiment in 1× HBS and 40 µl injected over all four flow cells. Following association, running buffer was passed over the sensor chip surface for 6 min to allow the measurement of complex dissociation. A subsequent 1 min injection of 50 mM NaOH permitted regeneration of the binding surface without decreasing the amount of immobilised ligand on the streptavidin chip surface. Prior to fitting curves, non-specific binding of analyte to the chip surface, represented as the sensorgram recorded in flow cell 1, was subtracted from all relevant datasets. The determination of association (k_a) and dissociation (k_d) rate constants was performed by non-linear fitting to a 1:1 (Langmuir) isotherm, from which equilibrium dissociation constants were subsequently calculated. Rate constants were calculated using data from seven different concentrations of deleted template (ranging from 20 nM to 1 µM) and for eight concentrations of wild-type templates (ranging from 50 nM to 5 µM). Global and local fittings were performed on sets and individual sensograms, respectively, using the BIAevaluation 3.0 software package (Biosensor, Stevenage, UK). Fits for all datasets are available on request.

Thermal melt analysis

Thermal melt profiles of the anti-genomic ODNs hybridising to mtDNA templates were obtained using 1 µM relevant oligodeoxynucleotides in a buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 14 mM β-mercaptoethanol and 150 mM KCl. Mixtures were heated to 90°C and allowed to anneal by slow cooling to room temperature. The mixture was split into two cuvettes (sample and reference). Melt profiles were measured at 260 nm using a Lambda Bio 20 UV/Visible spectrophotometer (Applied Biosystems, Cheshire, UK); the temperature of the sample cuvette was modulated by a PTP-1 Peltier System (Applied Biosystems). Data were collected and analysed using TempLab software (PE Biosystems, Cheshire, UK). Samples were heated at a rate of 0.5°C min^{-1} and the sigmoidal melt curve expressed as the first derivative (ΔA_{260}/time against unit time) to provide an accurate measurement of the melting temperature.

In vitro replication run-off assays

The capacity of various ODNs to inhibit replication run-off from single-stranded mtDNA templates was assessed essentially as described (8). For the 5 nt repeat deletion and the corresponding wild-type templates M13-tailed primers were designed to amplify synthetic templates by PCR, with each L strand primer carrying a 5′-biotin modification. This template preparation was found to increase the efficiency of template replication by the mitochondrial extract. The 118 bp products were generated as follows: *in vitro*-Delete was amplified using the pair M13 (L7451-bio) and M13 (H14508); *in vitro*-WT7502 with M13 (L7451-bio) and M13 (H7532); *in vitro*-WT14432

Table 2. Sequences of synthetic single-stranded DNA templates used in this study

Template designation	Corresponding mtDNA sequence	Sequence (5′→3′)[a]
DEL5	14448–14428 and 7506–7486	gaggagtatcctgagg CATGG aggccatggggttggc
WT5-3′	7522–7486	acctttttgaaaaagt CATGG aggccatggggttggc
WT5-5′	14448–14412	gaggagtatcctgagg CATGG gggtcaggggttgagg
DEL13	13475–13447 and 8482–8454	aatgctaggctgccaa TGGTGAGGGAGGT aggtggtagtttgtgt
WT13-3′	8498–8454	ttatttttatgggctt TGGTGAGGGAGGT aggtggtagtttgtgt
WT13-5′	13475–13431	aatgctaggctgccaa TGGTGAGGGAGGT tgaagtgagaggtatg

[a]The sequence indicated is antisense to the published L strand sequence (16), corresponding to the region of mtDNA indicated. Upper case indicates the deletion repeat sequence. Underlining indicates sequence complementarity to the appropriate anti-genomic oligomer.

was amplified with M13 (L14383-bio) and M13 (H14508). Exact primer sequences are available on request. Samples were subjected to 30 cycles of amplification using the following conditions: denaturation at 94°C for 1 min, annealing at 40°C for 1 min and extension at 72°C for 1 min; the final extension proceeded for 8 min. To generate common deletion and analogous wild-type templates, skeletal muscle DNA from a patient with the common deletion was amplified using primer pairs with 5′-biotinylated L strand primers (exact primer sequences are available on request). The deleted template carrying the 13 nt repeat sequence was amplified using L8285-5′bio and H13559; template containing WT13-3′ using L13260-5′bio and H13559; template containing WT13-5′ using L8285-5′bio and H8582. PCR conditions were 30 cycles of amplification with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; the final extension proceeded for 8 min. For all experiments biotinylated PCR products were purified (Qiaquick PCR purification kit; Qiagen Ltd) and either bound to streptavidin-coated magnetic beads (Dynal A.S.) or subjected to DNA sequencing to verify the product (ABI Big Dye terminator sequencing). Non-biotinylated single-strand mtDNA templates were recovered by ethanol precipitation following denaturation of double-stranded DNA with 100 mM NaOH.

For calculating inhibition efficiency, plates were read using a Storm 860 scanner (Molecular Dynamics) and analysed using ImageQuant software (Molecular Dynamics). Rectangular object boxes were drawn down the lengths of the lanes to detect all products. Reports showed the number of counts detected within the peak area. For some assays the inhibitory oligonucleotide produced a short stuttering around the inhibition site. These were counted together and the reports therefore gave a total number of counts for these products. The counts for the truncated species were corrected for size in relation to the full-length template. Once size corrected, the percentage of truncated product was calculated as [counts of truncated product/(counts of truncated product + counts of full-length product)] × 100. Intermediate sized non-specific extension products were not included. For those situations where a non-specific product was detected in the control extension assay at the same size as the specific product (see Fig. 5), the number of counts at this site was calculated as a percentage of the total number of counts in the full-length product. This percentage was used as an indication of background and deducted from the truncation product in the inhibition experiments.

RESULTS

Bridging oligodeoxynucleotides show intramolecular binding cooperativity

The immediate aim of these experiments was to determine whether bridging ODNs could be made with greater binding affinity for templates containing a known mtDNA deletion breakpoint than for either of the two related, but undeleted, wild-type templates (Fig. 1). For the initial experiments a template containing a five residue deletion breakpoint, previously reported to be pathogenic, was chosen (15). Although it would have been possible to design a standard 17mer oligodeoxynucleotide that would bind at physiological temperature with greater affinity to a template carrying this short deletion sequence than either of the corresponding wild-type templates, this template was chosen to test the feasibility of using a bridging oligomer to target deletion breakpoints.

A 35mer template was synthesised, incorporating the 5 nt repeat sequence and identical to the deleted molecule across the breakpoint (DEL5, Table 2). Two templates representing the corresponding wild-type molecule were also constructed (WT5-5′ and WT5-3′, Table 2). Both carried the 5 nt repeat sequence but were only identical to DEL5 before (WT5-5′) or after (WT5-3′) the repeat sequence. Biotinylated ODN-binding partners were then synthesised (Table 1). A bridging ODN was made with two stretches of nine residues complementary to the nucleotide sequence 5′ and 3′ to the 5 nt repeat sequence of DEL5, connected by two polyoxyethylene spacers ($9XX9^{DEL5}$). Two 9mers alone that were complementary either to the sequence 5′ ($9mer\text{-}5'^{DEL5}$) or 3′ ($9mer\text{-}3'^{DEL5}$) to the repeat were also synthesised. Finally, a molecule of 23 residues entirely complementary to DEL5 ($9\text{-}5\text{-}9^{DEL5}$) was made.

To determine the binding affinity of ODNs to the various templates, two biophysical methods were used. First, binding kinetics were studied using surface plasmon resonance technology. Secondly, melting curves were performed to assess thermal stability. Binding curves were produced by passing each of the three templates DEL5, WT5-3′ and WT5-5′ over the immobilised ODNs. To determine accurate rate constants, each template was injected at various concentrations. The resultant data were fitted to a simple 1:1 bimolecular

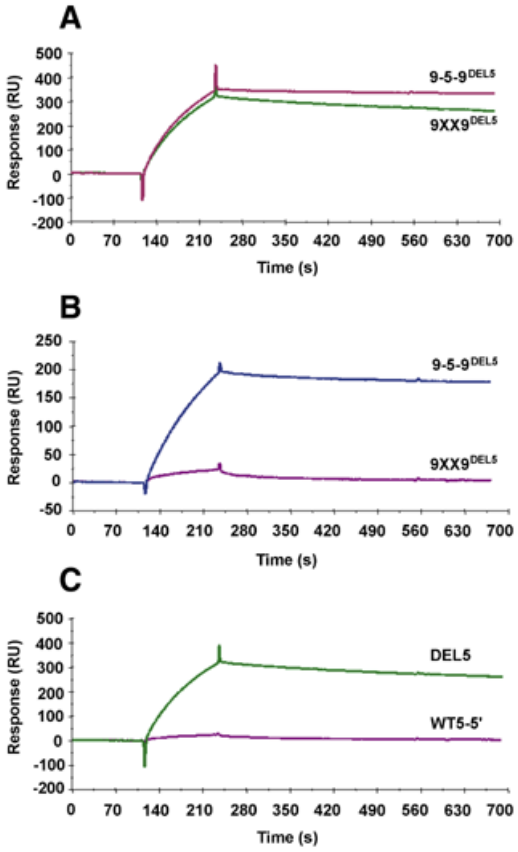

Figure 2. The bridging ODN 9XX9^{DEL5} has a greater binding affinity for the deleted template DEL5 than for the corresponding wild-type templates. Details of all kinetic experiments are given in Materials and Methods. A representative sensorgram is shown for each binding pair. (**A**) Sensorgram showing binding of analyte DEL5 (1 µM) to immobilised ODNs 9-5-9^{DEL5} and 9XX9^{DEL5}. Substitution of 5 nt, representing the repeat sequence at which the deletion occurs, with two Spacer 18 molecules only marginally reduces the binding affinity for the DEL5 template. (**B**) Sensorgram showing binding of wild-type template WT5-5′ (1 µM) to 9-5-9^{DEL5} and 9XX9^{DEL5}. As expected, the additional five residues of complementarity in ODN 9-5-9^{DEL5} induce an increased affinity for the wild-type template. (**C**) Sensorgram demonstrating the different binding curves produced when immobilised ODN 9XX9^{DEL5} was subjected to 1 µM DEL5 and 1 µM WT5-5′. Providing the extra nine residues of complementarity proximal to the five residue deletion breakpoint in template DEL5 dramatically increases the binding affinity of the bridging ODN 9XX9^{DEL5}.

interaction model (A + B ↔ AB). Equilibrium dissociation constants for each binding pair were calculated and are shown in Table 3. With DEL5 as analyte, a rapid association occurs with the totally complementary 9-5-9^{DEL5} and the complex slowly dissociates, as expected (Fig. 2A). Encouragingly, the bridging ODN 9XX9^{DEL5} showed binding kinetics which qualitatively looked similar to 9-5-9^{DEL5}, but on detailed analysis had a slightly weaker affinity (3.5×10^{-8} compared with 5.6×10^{-9} M, Table 3). However, when the binding affinity of DEL5 was determined for either of the 9mer ligands alone a difference of at least an order of magnitude was noticed when compared to the bridging ligand 9XX9^{DEL5}.

These data show that substitution of the five central complementary residues in 9-5-9^{DEL5} by two linker molecules only partially reduces the binding affinity for DEL5 template (6.25-fold). Linking 9mers 9mer-5′^{DEL5} and 9mer-3′^{DEL5}, however, increase the binding affinity of the resultant molecule for DEL5 by 9- and 25-fold, respectively (3.2×10^{-7} and 9×10^{-7} compared with 3.5×10^{-8} M). Figure 2B shows the binding curves produced when DEL5 is replaced by WT5-5′ as analyte. This template contains the 5 nt repeat and nine residues 5′ to the repeat that are common to the DEL5 template, but exhibits no complementarity to 9-5-9^{DEL5} or 9XX9^{DEL5} 3′ to the repeat (Table 1). As expected, 9-5-9^{DEL5} had a higher affinity than 9XX9^{DEL5} due to the extra five residues of complementarity. 9mer-5′^{DEL5} bound to template WT5-5′ with a similar affinity to the bridging ODN 9XX9^{DEL5} (3.9×10^{-7} compared with 2.9×10^{-7} M). To illustrate the differential binding affinities of 9XX9^{DEL5} for templates which would be present in the heteroplasmic state, Figure 2C compares binding curves with DEL5 and the higher affinity of the two wild-type templates, WT5-5′.

Linking two 9mers promotes an increase in thermal stability of the duplex

Thermal stability data were then determined by subjecting each binding pair to a melting curve as detailed in Materials and Methods. As shown in Table 3, these data broadly support the kinetic analyses. For several pairs, however, particularly when using the short 9mers, an accurate T_m could not be determined. The bridging ODN 9XX9^{DEL5} and template WT5-5′ had a similar melting profile to the 9mer-5′^{DEL5}:WT5-5′ pair. Similarly, ODN 9XX9^{DEL5}:WT5-3′ exhibited a comparable profile to the 9mer-3′^{DEL5}:WT5-3′ pair. These results are consistent with the bridging ODN binding only via one or other of the nine residues either side of the linker molecules. Substituting the wild-type templates for DEL5 produced an increase in T_m with 9XX9^{DEL5} (41–45°C compared with 56°C, Table 3), consistent with the addition of the nine residue complementary sequence proximal or distal to the duplicated sequence promoting stability of the complex.

Bridging ODN 9XX9^{DEL5} partially inhibits replication of DEL5 but not wild-type template

The goal of our anti-genomic strategy is to design a nucleic acid derivative that will selectively inhibit replication of mtDNA molecules with known deletions. We recently described an *in vitro* run-off replication assay that uses a crude preparation of mitochondrial DNA polymerase γ (8). Having shown that the 9XX9^{DEL5} construct has a higher affinity for the deletion template than either of the corresponding wild-type templates, it was of interest to determine whether this molecule could selectively inhibit replication of the deleted template under physiological conditions *in vitro*. Single-stranded template that reproduced either the region around the deletion breakpoint or wild-type templates were prepared by PCR. Run-off assays were performed as described in Materials and Methods. With the deleted template and a 100:1 molar excess of 9XX9^{DEL5}, 10–15% inhibition of extension of the deleted template was detected (Fig. 3, lanes 5 and 6). Conversely, using the same amount of the bridging ODN, no inhibition of extension is noted with the wild-type template incorporating WT5-3′ (*in vitro*-WT7502, Fig. 3, lanes 2 and 3) or WT5-5′ (data not shown).

Table 3. Kinetic and thermal melt parameters delineating binding of anti-genomic oligonucleotides to various templates containing the duplicated 5 nt sequence found at the deletion breakpoint

Immobilised oligonucleotide	Template DNA					
	DEL5		WT5-3′		WT5-5′	
	K_D (M)	T_m (°C)	K_D (M)	T_m (°C)	K_D (M)	T_m (°C)
9mer-3′^{DEL5}	9.0×10^{-7}	ND[a]	NP[b]	42	$>1 \times 10^{-6}$	ND
9mer-5′^{DEL5}	3.2×10^{-7}	ND	$>1 \times 10^{-6}$	ND	3.9×10^{-7}	42
9XX9^{DEL5}	3.5×10^{-8}	56	2.0×10^{-6}	ND	2.9×10^{-7}	42
9-5-9^{DEL5}	5.6×10^{-9}	74	1.3×10^{-7}	60	3.9×10^{-8}	64

[a]An accurate measurement could not be determined due to the low thermal stability of the complex.
[b]This experiment was not performed.

Bridging ODNs can span the 'common' deletion breakpoint site

These findings establish that bridging ODN 9XX9^{DEL5} shows a greater binding affinity for complementary templates than either of the two component 9mers alone. As anti-genomic molecules, however, bridging ODNs are of limited value at physiological temperatures when the repeat sequence comprises only a short 5 bp repeat. Nucleic acid derivatives could be designed to bind selectively to the deleted template by synthesising a molecule which, in addition to the 5 nt repeat, contains six or seven residues of complementarity to either side. The concept of bridging anti-genomic ODNs is more relevant when the repeat sequence flanking the deletion site is larger. The deletion initiating from the 5 bp repeat sequence is rare. A more typical example is the 4977 bp 'common' deletion, which involves two identical 13 bp repeat sequences. When resolved, a single copy of this 13 bp repeat remains in the deleted molecule (14). This 'common' deletion has been shown to underlie many cases of mitochondrial disease and has been reported to accumulate as an individual ages (13).

A bridging ODN 9XX9^{DEL13} (Table 1) was therefore designed to span nine residues of this common deletion and was compared in all experiments to the fully complementary ODN 9-9-9^{DEL13} (Table 1). Templates were also synthesised to repeat the surface plasmon resonance and thermal melt analyses (DEL13, WT13-3′ and WT13-5′, Table 2). The affinity of DEL13 template to 9-9-9^{DEL13} was 33-fold greater than for the bridging ODN (5.5×10^{-10} compared with 1.8×10^{-8} M, Fig. 4A and Table 4), however, the bridging ODN shows greater binding affinity for the DEL13 template than for template WT13-3′ (1.8×10^{-8} compared with 3.3×10^{-7} M, Fig. 4C and Table 4). Crucially, the binding differential is more marked between the fully complementary and bridging ODNs for the WT13-3′ template than for DEL13 (33-fold and 550-fold, respectively). Thermal melt data were also generated (Table 4). Melting curves were performed with the bridging ODN 9XX9^{DEL13} or 9-9-9^{DEL13} with DEL13 and the two wild-type templates WT13-5′ and WT13-3′. Melting of 9-9-9^{DEL13} from the wild-type templates occurred at a lower temperature than from the fully complementary DEL13, as expected. However, suprisingly, there was little measurable difference in the binding affinities between these pairs, a result that, whilst consistently repeated, was difficult to explain. A T_m for the DEL13/9XX9^{DEL13} pair of 51°C was determined, in comparison to ~40°C with either of the wild-type templates. Finally, run-off replication assays were performed on templates containing either DEL13 or WT 13-3′ sequence in the presence of 100-fold molar excess of either the fully complementary or bridging ODNs. The former, ODN 9-9-9^{DEL13}, was able to inhibit replication of both templates to a similar degree (Fig. 5A and B, compare lanes 3). However, although inefficient, the bridging ODN 9XX9^{DEL13} could partially inhibit run-off extension of template DEL13 only (Fig. 5A, compare lanes 2 and 3), confirming selective inhibition by the bridging ODN.

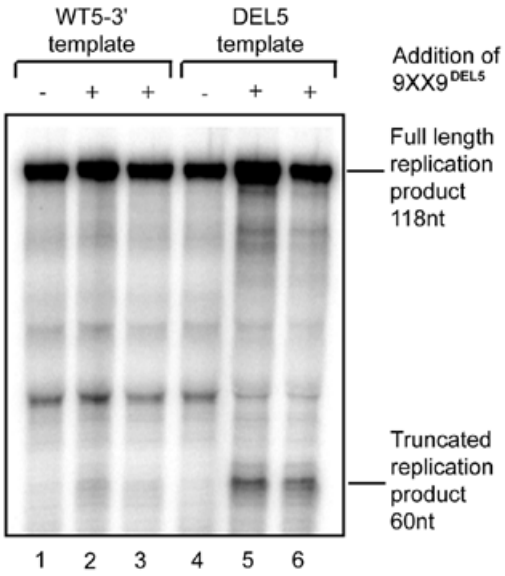

Figure 3. Binding of bridging ODN 9XX9^{DEL5} to single-stranded template DNA can partially inhibit replication by mitochondrial DNA polymerase γ. Template DNA molecules were prepared and run-off assays performed as detailed in Materials and Methods. Both templates incorporated the 5 nt deletion breakpoint but were identical in sequence either to the 6930 bp pathogenic molecule (DEL5) or the wild-type sequence (WT5-3′). For each template three run-off assays were performed, one without addition of the bridging ODN and duplicate assays with a 100-fold molar excess of bridging ODN. A full-length product of 118 nt was generated with wild-type template and was unaffected by addition of the bridging ODN (lanes 1–3). However, with the DEL5 template, a truncated product of ~60 nt was formed only on addition of bridging ODN (lane 4 versus lanes 5 and 6). Some truncated products are also seen in all assays as a consequence of incomplete synthesis by mtDNA polymerase γ.

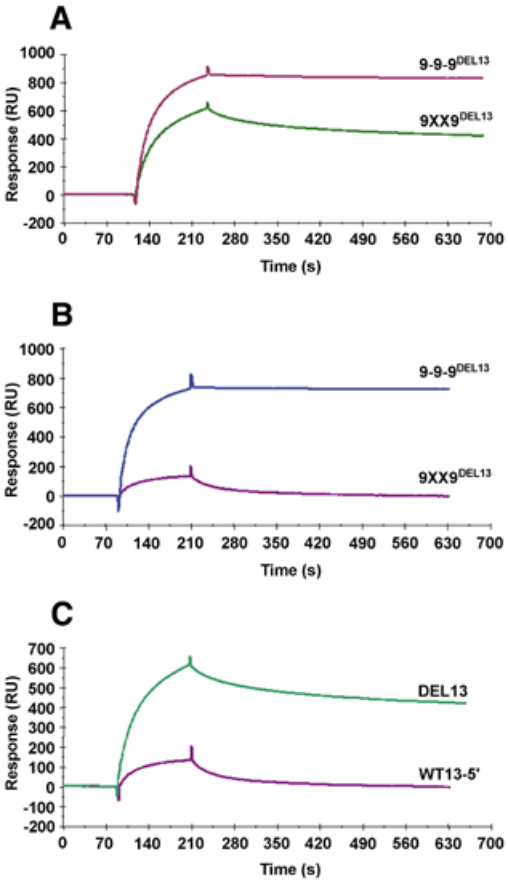

Figure 4. The bridging ODN 9XX9^{DEL13} has a greater binding affinity for the deleted template DEL13 than for the corresponding wild-type templates. Details of all kinetic experiments are given in Materials and Methods. A representative sensorgram is shown for each binding pair. (**A**) Sensorgram showing binding of analyte DEL13 (500 nM) to immobilised ODNs 9-9-9^{DEL13} and 9XX9^{DEL13}. Substitution of 9 nt spanning the majority of the 13 nt repeat sequence with two linker molecules only partially reduced the binding affinity for the DEL13 template. (**B**) Sensorgram showing binding of wild-type template WT13-5′ (500 nM) to oligos 9-9-9^{DEL13} and 9XX9^{DEL13}. As expected, the additional nine residues of complementarity in ODN 9-9-9^{DEL13} induces an increased affinity for the wild-type template. (**C**) Sensorgram demonstrating the different binding curves produced when immobilised bridging ODN 9XX9^{DEL13} was subjected to 500 nM DEL13 and WT13-5′. Providing the extra nine residues of complementarity proximal to the nine residue bridged sequence in template DEL13 dramatically increases the binding affinity of the bridging ODN 9XX9^{DEL13}.

DISCUSSION

Pathogenic mtDNA molecules are often heteroplasmic, with the mutated mtDNA sharing the same cell or tissue as the wild-type molecule. It has been reported on several occasions that the biochemical lesion can be exquisitely dependent on the ratios of the mutated and wild-type molecule. Attardi and colleagues previously showed, when studying a mt-tRNA$^{Leu(UUR)}$ mutation in cultured osteosarcoma trans-mitochondrial cybrids, that mitochondrial oxidation only becomes impaired with >94% mutation load (4). It has not been determined whether it is the amount of wild-type mtDNA, the total amount of mtDNA or indeed the level of heteroplasmy that determines the biochemical threshold. However, it is highly likely that by increasing levels of wild-type mtDNA the biochemical, and perhaps clinical, defect may be reverted. Consequently, inhibiting the replication of mutated mtDNA by targeting the single-stranded replication intermediate and allowing propagation of the wild-type could prove to be an effective therapy for disorders of the mitochondrial genome. Previously, we have shown that PNAs can be designed that will selectively inhibit replication of a template that carries a pathogenic single base pair mutation (8). The more common pathogenic deletions, however, present this more formidable test. As most deletions involve a repeat sequence, a single copy of the repeat is retained in the deleted molecule. We have therefore tried to design bridging ODNs that will span the deleted sequence but will bind to either side of the breakpoint in a cooperative manner, increasing their binding affinity. The data reported show that bridging ODNs can indeed show this characteristic. Our experiments focused on the use of two polyoxyethylene linker molecules separating two 9mer oligomers. Additional experiments have shown very similar kinetic and thermal stability parameters when one, two or three linker molecules separated the two 9mers (data not shown). Taken together, these results suggest that the flexible linker allows the two nonamers to bind freely to their complementary sequences and do not impart any steric constraints.

We have not optimised the size or type of nucleic acid derivative, but these data are proof of principle for selective targeting of deleted mtDNA. Data from the replication run-off assays show that in addition to altering binding affinities as judged by thermodynamic and kinetic parameters, the bridging ODNs can partially inhibit replication. The level of partial inhibition is low in comparison to the 70% levels of inhibition seen previously (8). It must be stressed, however, that our previous experiments used PNAs as anti-genomic agents. These molecules show higher binding affinity for complementary template molecules than their oligodeoxynucleotide counterparts and the complex exhibits greater thermal stability, which is dramatically destabilised by a single base pair mismatch (17,18). By using other nucleic acid derivatives, such as bridging PNAs or locked nucleic acid (LNA) molecules (19), this selective inhibition is highly likely to be increased. Indeed, preliminary data suggest that experiments with bridging PNA

Table 4. Kinetic and thermal melt parameters delineating binding of anti-genomic oligonucleotides to various templates containing the duplicated 13 nt sequence found at the breakpoint for the common deletion

Immobilised oligonucleotide	Template DNA					
	DEL13		WT13-3′		WT13-5′	
	K_D (M)	T_m (°C)	K_D (M)	T_m (°C)	K_D (M)	T_m (°C)
9XX9^{DEL13}	1.8×10^{-8}	50.9 ± 0.4 (n = 8)	3.3×10^{-7}	35–40 (broad melt)	3.8×10^{-7}	41
9-9-9^{DEL13}	5.5×10^{-10}	74.1 ± 0.3 (n = 7)	6.0×10^{-10}	69.5 ± 0.1 (n = 5)	2.6×10^{-9}	70

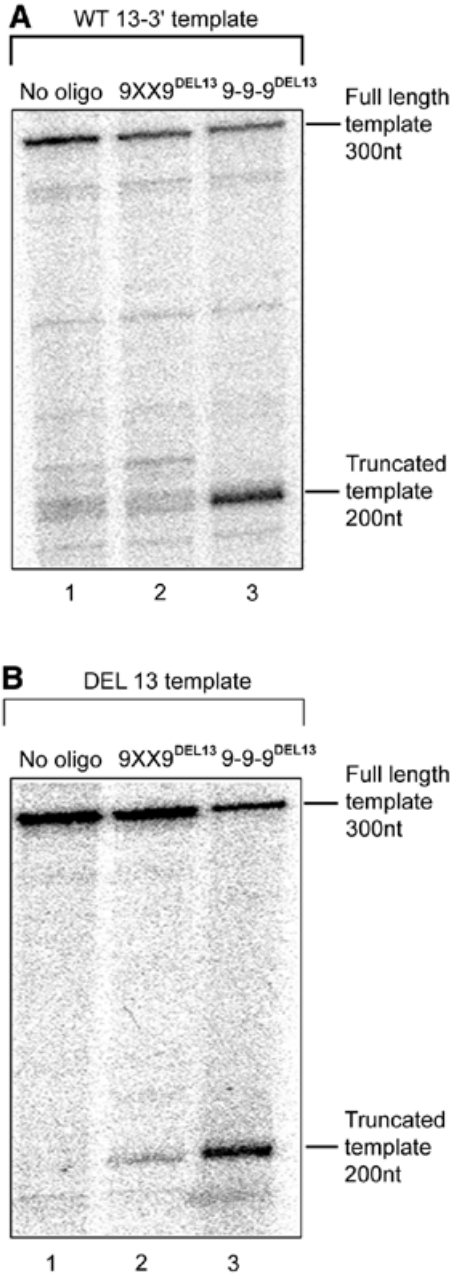

Figure 5. Binding of bridging ODN 9XX9^{DEL13} to single-stranded template DEL13 can partially inhibit replication by mitochondrial DNA polymerase γ. Template DNA was prepared and run-off assays performed as detailed in Materials and Methods. A full-length run-off product of 300 nt was formed in all assays. For the wild-type template WT 13-3′ no inhibition was caused by either 100- (**A**, lane 2) or 1000-fold molar excess of the linker oligomer 9XX9^{DEL13}. However, a 100-fold excess of this linker molecule inhibited ~9% run-off from deletion template DEL13 (**B**, lane 2), as evidenced by formation of a 200 nt truncation product. Inhibition by 100-fold excess of the fully complementary oligomer 9-9-9^{DEL13} was much more pronounced and was similar for both templates [compare (A) and (B), lanes 3].

molecules containing similar inert linker molecules do show higher levels of inhibition (data not shown).

Identifying anti-genomic molecules is only the initial phase for developing therapeutics for disorders of the mitochondrial genome. The anti-genomic agent must be internalised into cells and then pass across two mitochondrial membranes. Using various cell types, particularly human myoblasts, we have shown internalisation of PNAs from culture medium (9). In addition, we have been able to target PNAs to mitochondria by conjugating the PNA to a mitochondrial pre-protein that is cleaved from the PNA on import and by the addition of lipophilic cations (10). We now hope it will be possible to follow these initial experiments using ODNs with bridging PNAs or LNAs designed to target deleted mtDNA molecules as a first step towards providing an effective therapeutic for heteroplasmic disorders of the mitochondrial genome.

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