Selective inhibition of mutant human mitochondrial DNA replication *in vitro* by peptide nucleic acids

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Mitochondrial DNA (mtDNA) is the only extrachromosomal DNA in humans. It is a small (16.5 kb) genome which encodes 13 essential peptides of the respiratory chain, two rRNAs and 22 tRNAs. Defects of this genome are now recognized as important causes of disease and may take the form of point mutations or rearrangements[1–3]. There is no effective treatment for patients with mtDNA mutations[4]. In the majority of patients with mtDNA defects, both mutant and wild-type molecules are present in the same cell — a phenomenon known as intracellular heteroplasmy. In addition, in the presence of heteroplasmy there is a threshold whereby a certain level of mutant mtDNA is necessary before the disease becomes biochemically and clinically apparent[5–7]. Based on the presence of heteroplasmy and the recessive nature of these mutations, we believe it will be possible to treat patients by selectively inhibiting the replication of the mutant mtDNA, thereby allowing propagation of only the wild-type molecule. To confirm the validity of such an approach we synthesised peptide nucleic acids (PNAs) complementary to human mtDNA templates containing a deletion breakpoint or single base mutation, both mutations well documented to cause disease. Using an *in vitro* replication run-off assay under physiological conditions, the antigenomic PNAs specifically inhibited replication of mutant but not wild-type mtDNA templates. Furthermore, we have shown uptake of these PNAs into cultured human myoblasts. We believe that we have therefore established the potential value of antigenomic PNA therapy for patients with heteroplasmic mtDNA disorders.

Mitochondrial DNA is single-stranded during much of its replication process[8,9]. Whilst single-stranded, there is the opportunity for binding by sequence specific agents which could inhibit replication if not displaced by the mitochondrial DNA replisome. Initial experiments using sequence-specific oligonucleotides did not show selective inhibition of replication. We, therefore, did not pursue these experiments and decided to test our hypothesis by using peptide nucleic acids (PNAs). These polyamide, non-charged, nucleic acid derivatives obey Watson-Crick base pairing rules when forming a duplex with single stranded DNA[10–12]. They bind with greater affinity than the equivalent oligodeoxynucleotide to a complementary DNA strand. Furthermore, PNAs have also been shown to be substantially more resistant to degradation than their equivalent oligodeoxynucleotides in both extracellular fluid and intracellular environments[13].

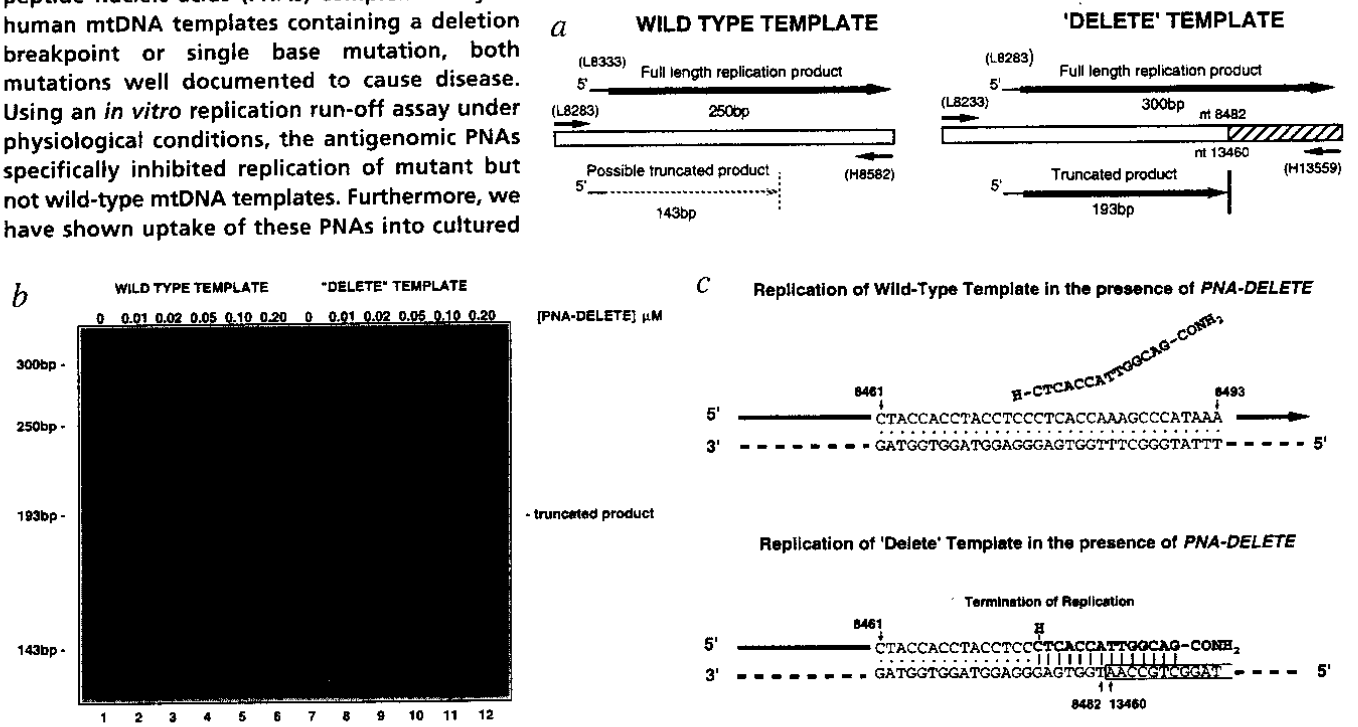


Fig. 1 Specific inhibition of mutant 'delete' template replication by a sequence-specific PNA. ***a***, Schematic representation of template production and sizes of expected replication products in the presence and absence of the 14 mer PNA, *PNA-DELETE*. PCR primers are indicated by small arrows, and their nucleotide positions as follows: L8233, nt 8233–8254; L8283, nt 8283–8302; L8333, nt 8333–8354; H8582, nt 8582–8565; H13559, nt 13559–13541. ***b***, Phosphorimage of the replication products generated in the presence of increasing concentrations (0–0.2 μM; 1:1–20:1 molar excess) of *PNA-DELETE*. Lanes 1–6, wild-type template; lanes 7–12, delete template. A truncated product generated by inhibition of replication due to *PNA-DELETE* is only visible in reaction lanes containing the mutant template (lanes 8–12). The product at 300 bp in lanes 1–6 is due to priming from L8283 carried over during the recovery of single-strand template. ***c***, Detailed representation of the PNA-induced inhibition of replication. The base composition of *PNA-DELETE* is shown in bold typeface. Template strands are the lower of the two sequences and are depicted by a dashed line, while the nascent strand is given above. Relevant nucleotide positions are also highlighted[30]. As only seven bases of *PNA-DELETE* are complementary to the wild-type template (upper panel), no inhibition of replication is seen. With the 'delete' template (lower panel), all fourteen bases of *PNA-DELETE* are complementary, and the *PNA-DELETE* binds across the deletion breakpoint (boxed region). Consequently, replication across this point is prevented by the binding of *PNA-DELETE*.

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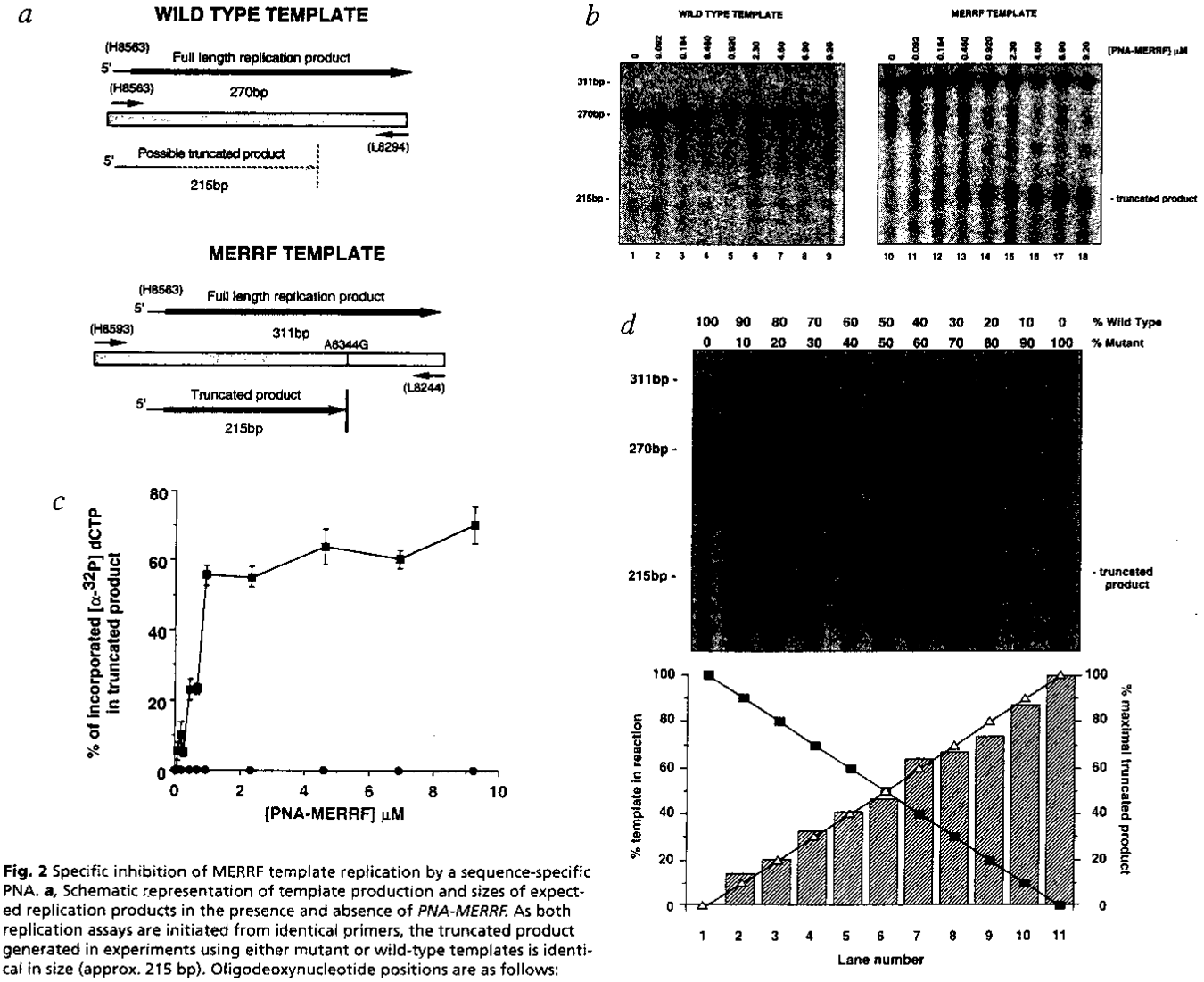


Fig. 2 Specific inhibition of MERRF template replication by a sequence-specific PNA. ***a***, Schematic representation of template production and sizes of expected replication products in the presence and absence of *PNA-MERRF*. As both replication assays are initiated from identical primers, the truncated product generated in experiments using either mutant or wild-type templates is identical in size (approx. 215 bp). Oligodeoxynucleotide positions are as follows: H8563, nt 8563–8544; H8593, nt 8593–8575; L8294, nt 8294–8314; L8244, nt 8244–8264. ***b***, Phosphorimage of the replication products generated in the presence of increasing concentrations (0–9.2 μM) of *PNA-MERRF*. Lanes 1–9, wild-type template; lanes 10–18, MERRF template. A truncated replication product is only apparent in the reactions containing the mutant template (lanes 11–18). The products at 270 bp in lanes 10–12 (MERRF template) are due to some pausing by the mitochondrial DNA polymerase γ towards the 3′ terminus of the single strand mutant template. ***c***, Incorporation of radiolabel into a 215-bp truncated replication product for both the wild-type (●) and mutant (■) templates (n=5, mean ± SD). This confirms the sequence-specific inhibition of only the mutant MERRF template. ***d***, Selective inhibition of MERRF mtDNA replication in the presence of wild-type template. Templates (total amount 10 ng) were mixed to mimic levels of mtDNA heteroplasmy ranging from 0–100% mutant mtDNA. In the presence of 4.6 μM *PNA-MERRF*, an increasing amount of truncated product is generated as the amount of mutant template in the reaction mix is increased. The histogram highlights the concomitant increase of incorporation of [α-^{32}P] dCTP into the truncated replication product, with the increasing levels of MERRF template (△) in the reaction mix. The % wild-type template in each replication reaction is shown (■).

To assess the potential value of PNAs in the treatment of patients with mtDNA deletions we used an *in vitro* replication run-off assay. We generated two templates, one wild-type and one encompassing the most frequent mtDNA rearrangement detected in patients, the 4977 bp 'common' deletion[14–16] (Fig. 1*a*). On the basis of published T^m for PNA:DNA heteroduplexes[17], a 14-mer peptide nucleic acid (PNA-DELETE) was synthesized complementary to the deletion breakpoint sequence (5′–CTGCCAATG-GTGAG–3′; ref. 16) in the mutant mtDNA template, whilst only the first seven bases of this PNA were complementary to the wild-type template. Inhibition of replication following addition of PNA-DELETE would result in the formation of a truncated replication product (Fig. 1*a*). All replication assays were initiated by the addition of a mitochondrial protein fraction containing mtDNA polymerase γ[18]. Using 10 ng of single-stranded human templates, the 14-mer PNA-DELETE specifically inhibited replication of the mutant template (>80% inhibition; K_i approx. 0.045 μM) and an appropriately sized truncated product was generated (Fig. 1*b*). There was no inhibition of replication of the wild-type template by PNA-DELETE (Fig. 1*b*, lanes 2–6).

Heteroplasmic tRNA point mutations are an important cause of mitochondrial disease. We, therefore, wished to determine if replication could be inhibited when the mutant mtDNA contained only a single point mutation. In view of the clinical importance of the heteroplasmic A8344G $tRNA^{Lys}$ (MERRF) mutation[19,20], further studies were focused on whether a PNA could selectively inhibit replication of a template containing this mutation. Templates were generated as detailed in the legend to Fig. 2*a*. An 11-mer PNA (*PNA-MERRF*) was synthesized which was complementary to the light strand MERRF sequence (5′ AGA-GAGCCAAC 3′: mutant base at nucleotide position 8344 shown in bold). Replication run-off from MERRF mtDNA templates was inhibited by up to 75% in the presence of *PNA-MERRF* (estimated K_i 0.8 μM; Figs. 2*b*,*c*) as shown by the formation of an appro-

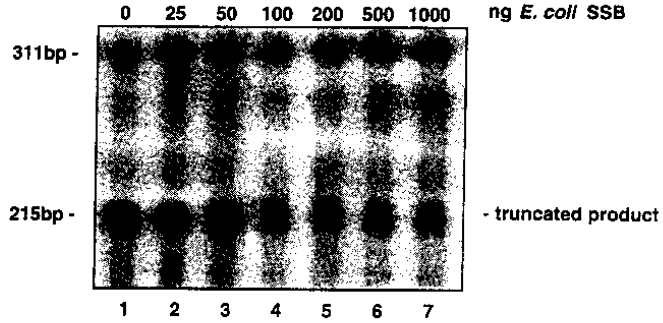
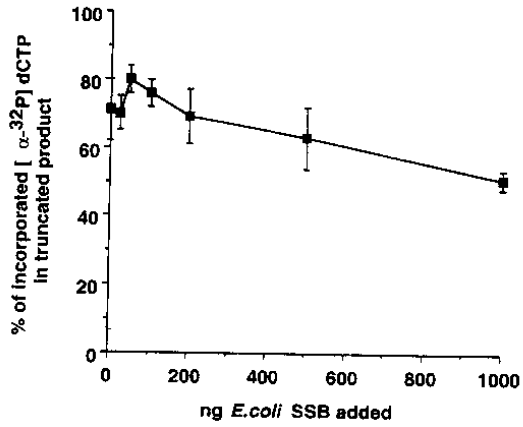


Fig. 3 Effect of *E. coli* SSB on inhibition of MERRF template replication by *PNA-MERRF*. A phosphorimage of the inhibition of MERRF template replication by 4.6 μM *PNA-MERRF* with increasing amounts of SSB (0–1000 ng) is shown. *PNA-MERRF* and SSB were added simultaneously to the mtDNA replication run-off assays. As shown in the graph, at the ratio of template:SSB that gives maximal stimulation of mitochondrial polymerase γ activity and saturation of binding (50 ng SSB, lane 3, phosphorimage), and at higher concentrations of SSB, there is still substantial (50–80%) inhibition of MERRF template replication by *PNA-MERRF* (n=3, mean±SD).



priately sized replication product. Identical concentrations of *PNA-MERRF* (up to 1,000-fold molar excess) did not inhibit replication of the wild type template (Figs. 2*b*,*c*).

To confirm this sequence-specific inhibition and to reflect the situation *in vivo*, defined ratios of wild-type and mutant templates were mixed and the replication run-off assay performed in the presence of 4.6 μM *PNA-MERRF*, a concentration producing near maximal inhibition. Whilst any truncated replication product generated from the wild type template would be the same size as that generated from the MERRF template (Fig. 2*a*), no product of the appropriate size was visible with 100% wild type template (Fig. 2*d*, lane 1). Furthermore, as the proportion of MERRF template in the mix was gradually increased, there was a concomitant increase in the formation of the truncated replication product (Fig. 2*d*).

Mitochondrial DNA replication involves extensive formation of single-stranded mtDNA. Mitochondrial single-stranded binding protein (SSB) may therefore influence the binding of an anti-genomic inhibitory agent such as a PNA. Human mitochondrial SSB has been purified to homogeneity from mitochondria and has been shown to have many properties analogous to the *E. coli* protein[21]. SSB stimulates DNA replication *in vitro* by mitochondrial DNA polymerase γ prepared from a variety of species[22–24]. To determine the effect of SSB on human mtDNA synthesis *in vitro*, increasing amounts of *E. coli* SSB were added to the run-off replication assay. At low salt concentrations, *E. coli* SSB stimulated the replication of both the delete and MERRF templates (data not shown) with maximal stimulation occurring at a molar ratio of 1:7.5 (mtDNA template:SSB tetramer), consistent with the average binding site of 46 nucleotides per SSB tetramer. This compares favourably to published estimates of between 33–65 nucleotides dependent on salt concentrations[25]. Using amounts of *E. coli* SSB where template was saturated, inhibition of mtDNA replication by *PNA-MERRF* was still maximal (Fig 3, lane 3). Even in the presence of a large molar excess of *E. coli* SSB, greater than 50% inhibition of replication was apparent in the presence of *PNA-MERRF* (Fig 3, lane 7). Thus, high concentrations of DNA-binding proteins such as SSB do not prevent the selective inhibition of replication of mutant mtDNA by the antigenomic agent *PNA-MERRF*.

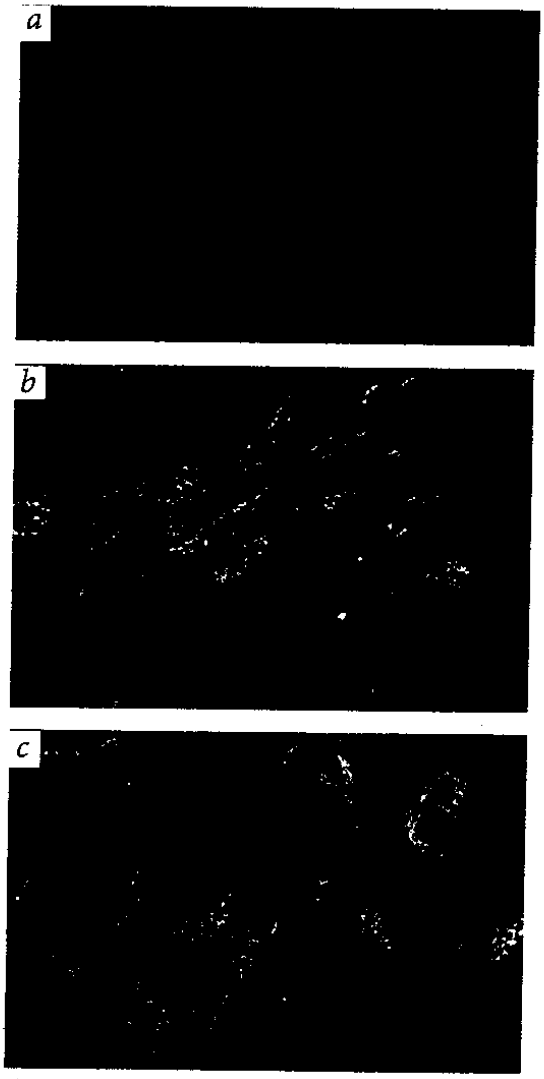


Fig. 4 Uptake of PNA into human myotubes. ***a***, 20 μM Biotin. ***b***, 20 μM DNA for 4 h. ***c***, 20 μM PNA for 4 h. Brightness and contrast were enhanced for image (*a*) to illustrate the cell profiles. Magnification x400.

Our *in vitro* studies have shown that we can selectively inhibit mtDNA replication in a sequence specific manner. Although this will enable the development of a strategy for treating patients with heteroplasmic mtDNA defects, there are many obstacles to overcome. The uptake of PNAs into cells, for example, has not been demonstrated and this is an essential step if ultimately they are to be targeted to mitochondria. To assess the ability of PNAs to be imported into human muscle cells in culture, we studied the uptake of biotin labelled DNA (DNA,14-mer) and PNA (PNA, 11-mer) into human myotubes. On exposure to 20 μM free biotin (Fig. 4*a*), no increase in fluorescence compared to control cells was noted (mean intracellular fluorescent pixel intensity relative to background (MIF/B mean ± SD: 1.14 ± 0.16, n=4)). However, myotubes exposed to both DNA and PNA showed extensive intracellular fluorescence within 30 min. The level of intracellular fluorescence increased over the subsequent 5.5 h (MIF/B at 6 h DNA 7.59 ± 1.59, n=8; PNA 4.21 ± 0.79, n=16) (Fig. 4*b*,*c*). Punctate intracellular staining became more diffuse after extended incubation, consistent with the PNA gaining access to the cytosol. Uptake of DNA into yeast[26] and mammalian[27] mitochondria has been achieved using DNA conjugated to mitochondrial precursor peptides. A similar approach is currently under investigation using peptide-PNA conjugates.

Methods

Patient samples. Total muscle DNA was extracted from patients heteroplasmic for either the 4977 bp 'common' deletion or the A8344G $tRNA^{Lys}$ (MERRF) mutation. To generate homoplasmic mutant MERRF template for *in vitro* studies, an 875 bp region of mtDNA encompassing the $tRNA^{Lys}$ gene was amplified using muscle DNA from a patient with the A8344G MERRF mutation and oligodeoxynucleotide primers L8154 (nt 8154–8171) and H9028 (nt 9028–9008). PCR products were subcloned into a pCRII plasmid vector (Invitrogen). Individual bacterial clones were isolated and plasmids sequenced[28] (Sequenase, USB) to identify those containing the A8344G mutation. Mitochondrial DNA from this patient also contained a common 9 bp deletion polymorphism[29].

Generation of single strand templates. Regions of mtDNA encompassing either the deletion breakpoint[15] or the mitochondrial $tRNA^{Lys}$ gene were amplified by PCR. All oligodeoxynucleotide primers (Figs 1*a*, 2*a*) were designed and named with reference to their nucleotide position given in the published sequence of human mtDNA[30]. Light (L) and heavy (H) refer to primers synthesized to generate light and heavy strand products respectively. In reactions to generate 'deletion' templates (Fig 1*a*), light strand primers were biotinylated; in reactions to generate $tRNA^{Lys}$ templates, heavy strand primers were biotinylated. Samples were subjected to 30 cycles of amplification using the following PCR conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min; the final extension proceeded for 8 min. Biotinylated PCR products were bound to streptavidin-coated magnetic beads (Dynal A.S.) and the non-biotinylated single-strand recovered by ethanol precipitation following denaturation of double-stranded DNA by 0.1N NaOH.

Peptide nucleic acids. PNAs were custom synthesized, purified and analysed by PerSeptive Biosystems (Framingham, MA). The two PNAs used in this study were: PNA-DELETE, H-CTCACCATTGGCAG-NH_2; *PNA-MERRF*, H-Lys-TCTCTCGGTTG-BIOTIN-NH_2. Lyophilized PNAs were dissolved in 0.1% trifluoroacetic acid and concentrations determined by measuring UV absorbance at 55 °C.

Isolation of a mitochondrial DNA polymerase activity. Bovine liver mitochondria were isolated using standard methods and purified on sucrose gradients. Bovine liver mitochondrial protein (2 g) was fractionated by chromatographic procedures as described[18] and all replication run-off assays were initiated by the addition of fraction III.

***In vitro* replication run off assays.** Single-strand templates were incubated in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 150 mM KCl, 1 mM ATP, 100 μM each of dATP, dGTP and dTTP, in the presence of 100nM specific priming oligodeoxynucleotide (Figs 1*a*, 2*a*). Samples were incubated at 70 °C for 3 min and cooled to 37 °C to allow hybridization of the oligodeoxynucleotide. Following the addition of PNA, 10 μM dCTP, 0.33 μM [α-^{32} P] dCTP (3,000 Ci/mmol) and 1.5 μg enzyme fraction, reaction mixtures were incubated at 37 °C for 60 min. Incubation was terminated by the addition of phenol. Replication products were ethanol precipitated and analysed on 6% denaturing polyacrylamide gels. Dried gels were exposed to a PhosphorImager cassette (Molecular Dynamics) and labelled products quantified using ImageQuant software.

Uptake of PNAs into human myotubes. Human myoblast cell lines were established from muscle biopsies performed on healthy individuals and were grown in Hams F10 medium supplemented with 20% fetal calf serum until 70% confluent and myoblast fusion was facilitated. After five days, mature myotubes were transferred to serum free medium for 12 hours before addition of 20 μM biotin , 20 μM DNA, or 20 μM PNA at 37 °C for a variable time period. Cells were fixed in 2.5% paraformaldehyde for 15 min, permeablised with 0.5% Triton in 2.5% paraformaldehyde and labelled with streptavadin fluorescein (1:50 dilution, Sigma) for 1 h at room temperature. Images were collected with a BioRad MRC 600 confocal laser microscope with fixed laser and photomultiplier parameters. Digital image analysis was performed (COMOS).

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