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Lack of RNA-DNA oligonucleotide (chimeroplast) mutagenic activity  
in mouse embryos.

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33 **Abstract**

34 There are numerous reports of the use of RNA-DNA oligonucleotides (chimeraplasts) to  
35 correct point mutations *in vitro* and *in vivo*, including the human apolipoprotein E gene.  
36 Despite the absence of selection for targeting, high efficiency conversion has been reported.  
37 Although mainly used to revert deleterious mutations for gene therapy applications,  
38 successful use of this approach would have the potential to greatly facilitate the production of  
39 defined mutations in mice and other species. We have attempted to create a point mutation in  
40 the mouse apolipoprotein E gene by microinjection of chimeraplast into the pronuclei of 1-  
41 cell mouse eggs. Following transfer of microinjected eggs we analysed 139 E12.5 embryos,  
42 but obtained no evidence for successful conversion.

43

44 **Keywords:** gene targeting, pronuclear injection, chimeraplast, oligonucleotide,  
45 mutagenesis, SNP

46

47 **Introduction**

48 With the availability of the finished human genome sequence and the draft mouse genome  
49 sequence, the priority is now the analysis of gene function. Important goals are the  
50 determination of the phenotypes of animals that have deficiencies in every gene (Nadeau et  
51 al, 2001) and, at another level, determination of the functional significance of naturally  
52 occurring DNA sequence variation, most of which is in the form of single nucleotide  
53 polymorphisms. For these ambitions to be realised, the efficiency of the generation and  
54 analysis of mutants is critical. Mice are the pre-eminent system for experimental mammalian  
55 genetics, and various strategies are in place for high-throughput mutagenesis of mice  
56 (Zambrowicz et al, 1998; Chen et al, 2000; Munroe et al, 2000; Hrabe de Angelis, 2000;  
57 Nolan et al, 2000; Wiles et al, 2000). These approaches are very powerful but they all have  
58 drawbacks: not least, they are all based on random events and there is no possibility of  
59 designing the mutation. Conventional gene targeting in ES cells is directed, permitting  
60 precisely designed mutations to be made in mice. Various refinements have streamlined the  
61 creation of mutants by gene targeting (Eggan et al, 2002; Seibler et al, 2003; Valenzuela et al,  
62 2003), but nevertheless, the process is technically demanding and remains relatively  
63 inefficient. The ideal approach to complement these established techniques would be highly  
64 efficient directed mutagenesis of embryos, applicable not only to mice, but also to other  
65 species.

66 A number of laboratories have reported success in correcting single base mutations in a  
67 variety of target genes using synthetic oligonucleotides (e.g. Alexeev et al., 1998, 2000;  
68 Bandyopadhyay et al., 1999; Cole-Strauss et al.,1996; Kren et al., 1997, 1998, 1999;  
69 Tagalakis et al., 2001). These studies have been undertaken with the ultimate goal of gene  
70 therapy applications, and typically the mutations targeted were reversion mutations. In most  
71 of these studies, the effective reagents used were synthetic self-complementary  
72 oligonucleotides known as RNA-DNA oligonucleotides (RDOs) or chimeraplasts, that are  
73 composed of DNA and 2'-O-methyl RNA segments, and which have terminal hairpins and a  
74 GC clamp to aid stability. Such synthetic oligonucleotide reagents have been found to be  
75 capable of targeting specific mutations in a variety of systems, including a variety of  
76 mammalian cells *in vitro*; liver, muscle and melanocytes *in vivo* as well as plants, and yeast.  
77 We have used chimeraplasts to target changes to human apolipoprotein E (Tagalakis et al,  
78 2001). In cultured cells, we have converted (at best) over 50% of gene copies and have  
79 rigorously validated these conversions showing that they are specific, stable, heritable and  
80 functional.

81 Successful use of synthetic oligonucleotides to target mutations directly in mouse embryos  
82 would dramatically speed up the production of specific mutant strains of mice because it  
83 would remove the need to build targeting vectors, ES cell culture and much else. For these  
84 reasons we have tested whether gene targeting can be accomplished in mouse embryos by  
85 microinjection of a chimeraplast.

86

## 87 **Materials and Methods**

### 88 **Pronuclear microinjection**

89 Microinjection was done using standard procedures (Hogan et al, 1994). C57BL/6 x CBA/Ca  
90 F<sub>1</sub> mice were used throughout, apart from CD1 vasectomised males. Females were  
91 superovulated with 5IU PMS and 5IU hCG; embryos were recovered in M2 medium and  
92 cultured in M16 in 5% CO<sub>2</sub>, under oil. The chimeraplast was dissolved in 10mM Tris-HCl  
93 pH8.0, 0.1mM EDTA. One pronucleus of each embryo was microinjected, and any embryo in  
94 which the injected pronucleus was not observed to swell was discarded. Following  
95 microinjection the embryos were cultured overnight. For assessment of chimeraplast toxicity,  
96 2-cell embryos were transferred to fresh M16 and cultured for a further three days, at which  
97 time they were evaluated. Normal blastocysts were those which had not fragmented, which  
98 had a single discrete blastocoel and which had expanded to at least fill the zona pellucida. To  
99 test for chimeraplast-mediated mutagenesis, 2-cell embryos were transferred (average 28 per

100 mouse, range 18-35); they were transferred to one oviduct of pseudopregnant foster mothers  
101 under avertin anaesthesia (Papaioannou & Fox, 1993). These experiments were performed  
102 fully in compliance with current UK legislation.

103

#### 104 Screening for the mutation

105 The region of the mouse ApoE gene that includes the two sites that are most polymorphic in  
106 man was amplified by PCR using primers cgcacatggaggatctacgc and ggcacctgtctccaccagag.  
107 PCR conditions were 75mM Tris-HCl (pH8.8 at 25°C), 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% Tween 20,  
108 1.5mM MgCl<sub>2</sub>, 0.1mM dNTPs, 10% DMSO, 0.6µM each primer and 0.04u/µl Taq  
109 polymerase. Following an initial denaturation for 5 mins at 95°C, 36 cycles of 94°C, 62°C and  
110 72°C (40 sec each) were performed with a final extension of 8mins at 72°C. We sequenced  
111 PCR products from this region of the ApoE gene from wild-type mice from our colony, and  
112 the sequences obtained corresponded to the published sequence.

113 DNA was prepared from E12.5 embryos by digesting embryo fragments (whole tail) in 100µl  
114 of 50mM KCl, 10mM Tris-HCl pH8.3 at 25°C 10µg/ml gelatine, 0.045% Igepal CA-630,  
115 0.045% Tween 20, 0.5mg/ml proteinase K at 55°C overnight. 7µl of the crude lysate was used  
116 per 25µl PCR reaction after heat inactivation of the proteinase K. For digestion with HinP1I,  
117 7µl of unpurified PCR products were mixed with an equal volume of H<sub>2</sub>O and 1µl of HinP1I  
118 (10u/µl) was added. Following overnight digestion, the DNA was run on 20%  
119 polyacrylamide gels (Novex) in TBE and stained with SYBR green I.

120

#### 121 **Results**

122 We selected apolipoprotein E (ApoE) as a test target for targeted mutation in mouse because  
123 we had previously achieved high level conversion of human ApoE genomic and cDNA  
124 targets in cultured cells and in transgenic mouse tissue (Tagalakis et al, 2001). The three  
125 common variants of human ApoE are ApoE2, ApoE3 and ApoE4. ApoE3 is the most  
126 frequent; the ApoE2 and ApoE4 alleles differ by virtue of single nucleotide polymorphisms  
127 that cause amino acid sequence changes. ApoE2 and ApoE4 are risk factors for  
128 atherosclerosis and Alzheimer's disease, respectively. We designed a C→T transition  
129 mutation that would simultaneously generate an RFLP and an R150C change in mouse ApoE  
130 protein (figure 1a), creating a SNP that is equivalent to the human ApoE2 SNP. A  
131 chimeraplast was synthesised to create this mutation (figure 1b) and was microinjected into  
132 the pronuclei of 1-cell mouse embryos.

133 For the production of transgenic mice, the volume of DNA injected into pronuclei is typically  
134 about  $2 \times 10^{-9}$  ml, and the optimum concentration of DNA is around  $2 \mu\text{g/ml}$ ; injection of DNA  
135 at concentrations above this significantly reduces the frequency of development of mice to  
136 term (Brinster et al, 1985). For a typical transgene of 10kb, this corresponds to about 350  
137 molecules of the construct; the chimeraplast is 68nt long, so a  $2 \times 10^{-9}$  ml injection would  
138 contain about  $10^5$  molecules. Double stranded DNA ends are recognised as a sign of  
139 chromosome damage; depending on the cell type and extent of the damage, the response to  
140 such damage may be cell cycle arrest, or apoptosis. To test whether chimeraplasts are toxic to  
141 preimplantation embryos we titrated the concentration of chimeraplast injected into pronuclei  
142 (0, 0.25, 2.5 and  $25 \mu\text{g/ml}$ ), and embryos that survived the immediate trauma of injection were  
143 cultured for 4 days. Development to normal blastocyst was significantly reduced for embryos  
144 injected with buffer or with chimeraplast, but there was no significant difference between the  
145 chimeraplast and buffer injected embryos (figure 2). This shows that the major deleterious  
146 effects of injection are independent of the injected DNA, and are probably due to physical  
147 damage. For subsequent experiments we injected the DNA at  $2.5 \mu\text{g/ml}$ .

148 To test for mutagenic activity of the chimeraplast, we injected it into pronuclear mouse eggs,  
149 transferred them into oviducts of pseudopregnant foster mothers, and harvested embryos 12  
150 days after transfer. A total of 662 eggs were injected, 420 of which developed to 2-cells and  
151 were transferred. 139 embryos were recovered at E12.5; DNA was extracted from each  
152 embryo, amplified and digested with HinP1I (figure 1c). None of the embryos gave the  
153 diagnostic 68bp fragment, and in other respects the restriction patterns were exactly as  
154 predicted (e.g. figure 3).

155

## 156 **Discussion**

157 All of the vasectomised males used to generate the recipients were confirmed to be sterile by  
158 test matings, some of the embryo recipients were carrying very large numbers of foetuses (up  
159 to 17), and some recipients were not pregnant. In mice, embryos do not move from one  
160 uterine horn to the other. The embryo transfers were unilateral, and every foetus recovered  
161 was in the ipsilateral uterine horn; there was no sign of implantation in the contralateral  
162 uterine horns. For these reasons we are confident that all of the recovered foetuses were  
163 derived from the eggs that had been injected and transferred, and were not from accidental  
164 fertile matings.

165 Some recent publications (Albuquerque-Silva et al., 2001; van der Steege et al., 2001;  
166 Taubes, 2002) have highlighted the inconsistency of oligonucleotide-directed targeted  
167 mutation. Various possible reasons for this have been proposed, including inefficient delivery  
168 to the cell nucleus, degradation, toxicity, poor reagent quality and differential accessibility of  
169 different target genes. We circumvented many of these concerns: the ApoE gene was chosen  
170 because the human homologue has been successfully targeted; direct microinjection  
171 eliminates delivery problems as  $>10^5$  molecules were injected per embryo; the chimeraplast  
172 gave a discrete band and showed no signs of degradation by gel electrophoresis (figure 4);  
173 and finally, the rate of development to blastocyst and to E12.5 of eggs microinjected with  
174 chimeraplast was incompatible with a toxicity effect.

175 The reason for our failure to obtain targeted conversions is not clear. We believe that it is not  
176 feasible to inject much more chimeraplast DNA without incurring significant  
177 postimplantation embryo losses. It has been proposed that chimeraplast activity is dependent  
178 on mismatch repair mechanisms. It is possible that preimplantation embryos lack the  
179 necessary mismatch repair activity; e.g. 1- to 4-cell stage rat embryos appear not to express  
180 Msh2 mRNA (Harrouk et al, 2000), but on the other hand, they may contain Msh2 protein of  
181 maternal origin. If not, it may be possible to stimulate chimeraplast-mediated mutagenesis by  
182 coinjection of Msh2 protein or components that may be lacking. Alternatively the use of a  
183 different chemistry may promote conversions in preimplantation embryos. There have been  
184 reports of targeted mutagenesis mediated by various types of single-strand oligonucleotides  
185 in yeast and/or cultured mammalian cells, including oligonucleotides that contain a triplex-  
186 forming region (Culver et al, 1999), those protected with phosphorothioates (PTO) (e.g.  
187 Kenner et al, 2002; Lu et al, 2003), locked nucleic acids (LNA) (Parekh-Olmedo et al, 2002)  
188 or including peptide nucleic acids (PNA) either conjugated with DNA oligonucleotides or as  
189 an adjuvant (Rogers et al, 2002). The PTO and LNA modifications probably function to  
190 stabilise the oligonucleotides by preventing exonuclease digestion; the triplex-forming  
191 oligonucleotides and PNA are thought to improve targeting by enhancing the interactions  
192 between the oligonucleotide and their targets. As yet the efficiencies obtained with single-  
193 strand oligonucleotides have not been high, but a number of factors have been reported to  
194 influence the efficiency of conversion. These include which strand is targeted with respect to  
195 the direction of transcription (Liu et al, 2002a; Pierce et al, 2003), transcriptional activity of  
196 the target (Liu et al, 2002; Igoucheva et al, 2003; Brachman & Kmiec, 2004), replication  
197 status of the target (Brachman & Kmiec, 2004) and single strandedness of the targeting  
198 oligonucleotide (Radecke et al, in press). Treatments that have been reported to increase the

199 efficiency of conversion include treatment of the cells with DNA damaging agents (Ferrara &  
200 Kmiec, 2004; Ferrara et al, 2004), modification of histone acetylation (and thereby chromatin  
201 structure; Parekh-Olmedo et al, 2003) and expression of normal or modified recombination  
202 proteins (Lio et al, 2002b, 2004). Because of the great attractiveness of gene repair for gene  
203 therapy, work is likely to continue in this direction, at least for somatic cells, and it is possible  
204 that this will result in improvements. Should significant progress be made in this direction, it  
205 may be worthwhile revisiting the possibility of oligonucleotide-mediated mutagenesis in  
206 preimplantation embryos. In this context it is obvious that any modified treatment regime  
207 must be compatible with normal development to term, as well as efficient mutagenesis of  
208 most or all targets. A further important consideration for any targeted mutagenesis  
209 programme is that second site mutations caused by the treatment should be kept to a  
210 minimum. While it may be possible to breed out unlinked second site mutations, it is likely  
211 that treatments such as the deliberate induction of DNA damage would give rise to sufficient  
212 random mutations to confound the analysis of phenotype.

213

#### 214 **Conclusions**

215 Whatever the reason for the apparent lack of chimeraplast mutagenic activity in mouse  
216 embryos, at present this technology is not suitable for large scale targeted mutagenesis  
217 programmes. Clearer understanding of the mechanisms of oligonucleotide-mediated targeted  
218 mutagenesis and the development of methods to increase the efficiency may, in the future,  
219 make this approach feasible.

220

#### 221 **Acknowledgements**

222 This work was funded by a Sir Henry Wellcome Commemorative Award from the Wellcome  
223 Trust (067727). Correspondence and requests for materials should be addressed to Paul  
224 Simons (simons@rfc.ucl.ac.uk).

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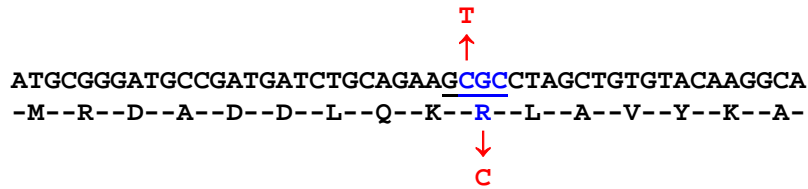
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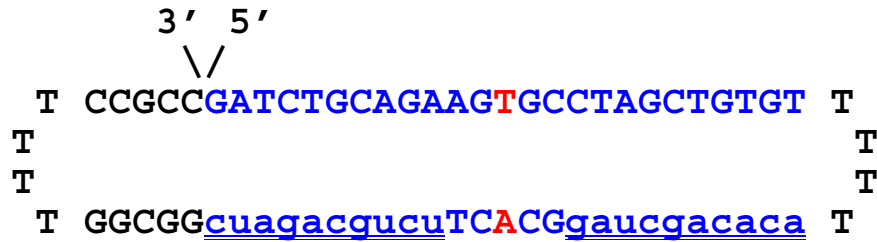
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**Figure 1**

**A**



**B**



**C**

```

ccgacatggaggatctacgcaaccgactcgggcagtaccgcaacgaggtgcac
accatgctgggcccagagcacagaggagatacgggcgcgggctctccacacacct
gcgcaagatgcgcaagcgcttgatgcgggatgccgatgatctgcagaagcgcc
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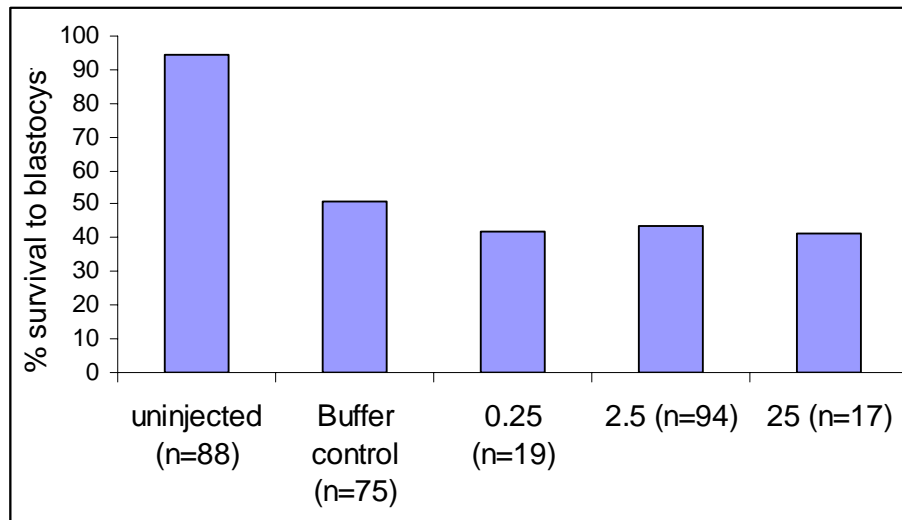
**A: Design of the mutation.**

The wild-type DNA and peptide sequence are shown. The target codon is shown in blue, and the targeted DNA sequence alteration is indicated in red; similarly the wild-type and mutant amino acids are shown. To test for successful mutation, we made use of the HinP1I restriction site (underlined) that overlaps the target codon; the wild-type sequence possesses a restriction site; the mutation was designed to destroy this site.

**B: Structure of the chimeraplast.** The sequence that is homologous to the mouse ApoE target is shown in blue, with the bases mismatched with the target (and corresponding to the intended sequence change) shown in red; DNA residues are shown in UPPERCASE, 2'-O-Me-RNA residues are shown in lowercase; the GC clamp and T residues of the hairpin loops are shown in black.

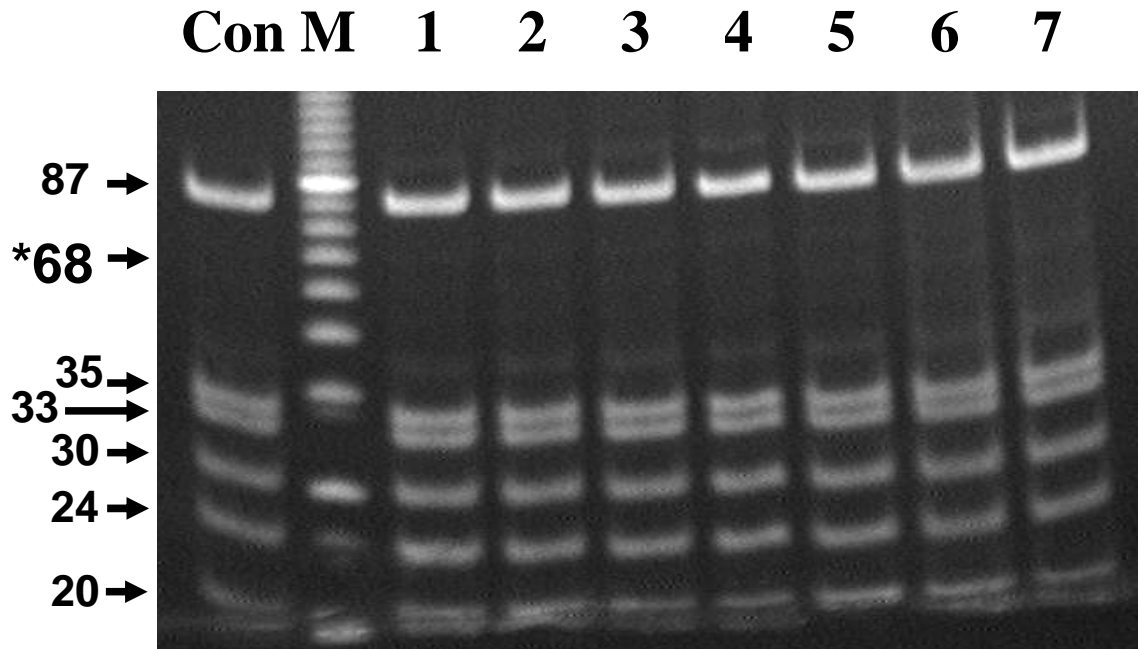
**C: PCR-RFLP analysis.**

The complete sequence of the wild-type PCR product is shown, with the targets of the PCR primers underlined, HinP1I sites are double underlined, and the target nucleotide is shown in red. The diagnostic restriction site is flanked by sites 33bp upstream and 35bp downstream. The wild-type allele is predicted to give fragments of 87, 35, 33, 30, 24, 20, 9, 7 and 6bp in length; mutation would cause loss of the 35 and 33bp fragments and a new 68bp fragment.



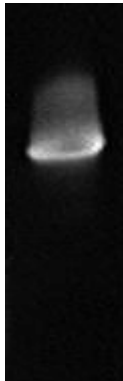
**Figure 2** Survival of embryos following injection of the chimeraplast.

Pronuclear mouse embryos were injected with chimeraplast at 0.25, 2.5 or 25 ng/ $\mu$ l, or with buffer alone; the embryos were cultured overnight. The following day, the lysed embryos were discarded, and the remaining 2-cell embryos were transferred to fresh medium (only 1/294 embryos had failed to divide). Control uninjected 2-cell embryos were also transferred to fresh medium. Following a further 3 days in culture, the normal blastocysts were counted. Chi-squared analysis shows that injections of buffer or 2.5 $\mu$ g/ml chimeraplast increased the frequency of abnormal development significantly ( $P < 0.001$ ;  $\chi^2 = 40.336$ , 1 d.f. and  $P < 0.001$ ;  $\chi^2 = 53.811$ , 1 d.f., respectively); the rates of normal development of chimeraplast-injected and control buffer-injected eggs were indistinguishable (for 2.5 $\mu$ g/ml chimeraplast,  $P = 0.361$ ;  $\chi^2 = 0.833$ , 1 d.f.)



**Figure 3** Representative screening results.

ApoE PCR products from chimeraplast-injected embryos recovered at mid-gestation (E12.5) were digested with HinP1I to screen for the introduction of the C→T mutation. Lanes 1-6 show representative patterns from such embryos. The mutant allele would give a new 68bp fragment (\*), with the 35 and 33bp fragments being depleted. We obtained no evidence for successful mutation in any of the 139 embryos analysed. The lane labelled Con shows the pattern obtained from an untreated wild-type mouse; lane M contains molecular weight standards (Invitrogen 10bp ladder).



**Figure 4**      **Intactness of the chimeraplast.**

The chimeraplast was run on a 15% polyacrylamide gel and stained with ethidium bromide. The bulk of the material migrated in a discrete band; the higher molecular weight smear is typical of chimeraplasts and is probably caused by intermolecular base pairing.